Skin Autofluorescence and Atherosclerosis

M.J. Noordzij

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Mijn kind,

Wees eerlijk, recht door zee.
Wanneer je iets belooft, kom dat dan meteen na.
Wanneer je ergens aan begint, maak het getrouw af.
Wanneer je iets doet, doe dat zo goed als je kunt.

Dit werd mij voorgeleefd door mijn lieve oma.
Wat ik van haar leerde, heb ik steeds bewaard in mijn hart.

Aan haar draag ik dit proefschrift op.

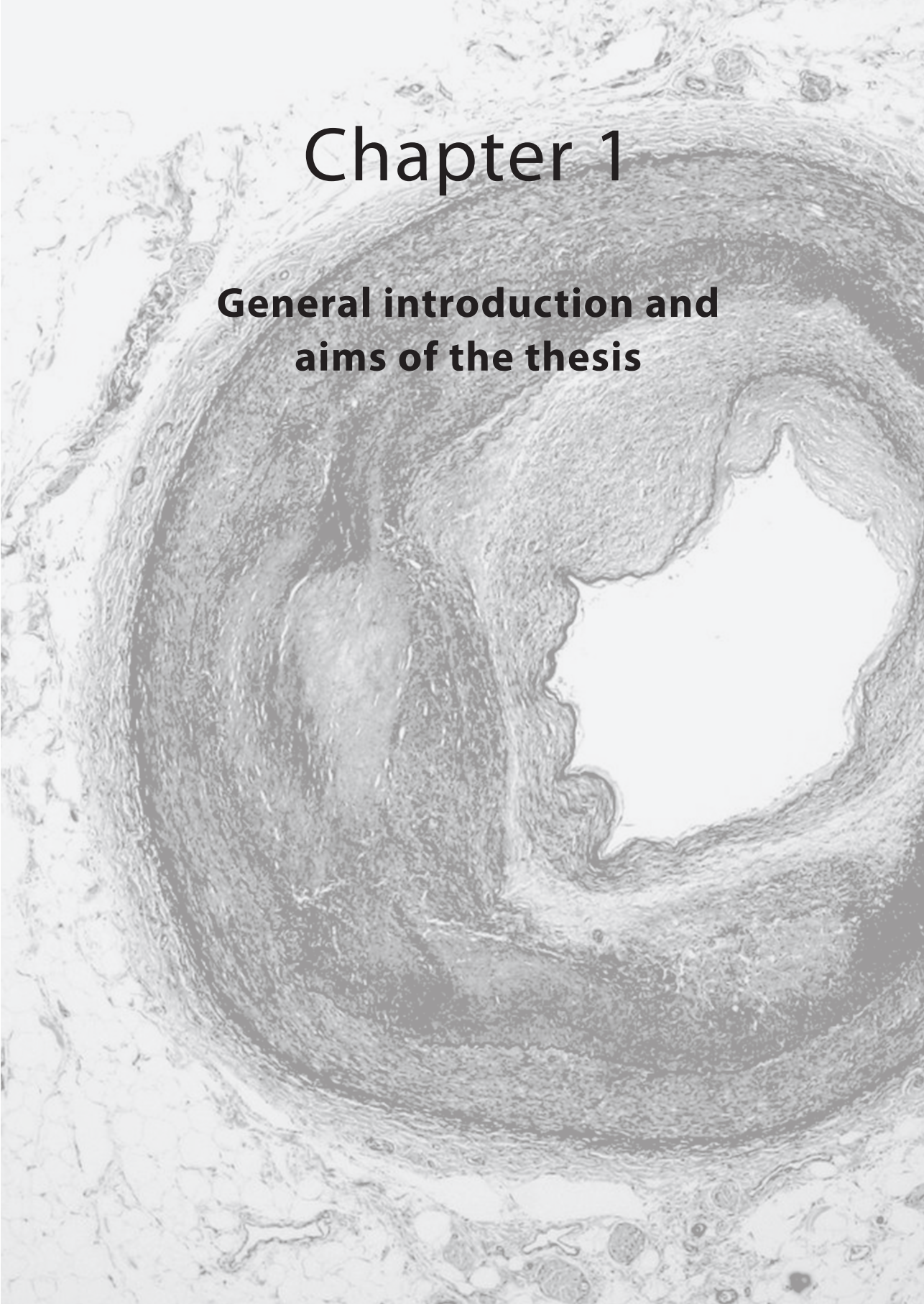
Contents

PART I

INTRODUCTION TO ADVANCED GLYCATION END PRODUCTS AND SKIN AUTOFLUORESCENCE

Chapter 1

General introduction and aims of the thesis



ADVANCED GLYCATION ENDPRODUCTS

What are advanced glycation endproducts (AGE), and how are they formed? Basically, AGE are the end products of a complex and diverse process of irreversible binding of sugars to other molecules in the body. The binding of sugar is called glycation and especially affects certain amino acids (like lysine).

Several pathways can lead to the formation of AGE (figure 1). The classical pathway is the Maillard reaction, called after the food chemist M. C. Maillard who first described the process in 1912. The first step is non-enzymatic glycation by the covalent binding of the carbonyl group of reducing sugars to free amino groups of a protein, forming an instable Schiff base. Secondly, a more stable intermediate Amadori product is formed by re-arrangement of the Schiff base by move of the hydrogen atom, leaving a ketone. One of the most well-known Amadori products is HbA1c, glycated haemoglobin, which is widely used to monitor diabetes regulation. Only a small part of the relatively stable Amadori products undergoes further oxidative reactions resulting in irreversible AGE. The best known representatives are pentosidine, carboxymethyllysine (CML) and pyrraline. The formation of AGE by this Maillard reaction is slow and concentration dependent and accelerates under conditions of hyperglycemia. Therefore, formation of AGE by the Maillard reaction is increased in diabetes mellitus.[1,2]

Besides this slow pathway of AGE formation it has now been recognised that, especially intracellularly, rapid formation of AGE may occur via reactive carbonyl compounds like glyoxal, methylglyoxal and 3-deoxyglucosone. Methylglyoxal leads to formation of AGE such as carboxyethyllysine (CEL) and methylglyoxal-derived hydroxyi midazolone (MG-H1), while glyoxal and 3-DG may result in formation of CML and pyrraline. The formation of reactive carbonyl compounds is mainly the result of oxidative stress.[3,4]

Peroxidation of polyunsaturated fatty acids to lipid peroxides may also modify proteins and result in so-called advanced lipoxidation endproducts (ALE): the lipid peroxides are then transformed to reactive carbonyl compounds which then again may react with amino acid residues.[5] This may also occur on nucleic acids. Thus, AGE formation may result from oxidative/carbonyl stress besides hyperglycemia.

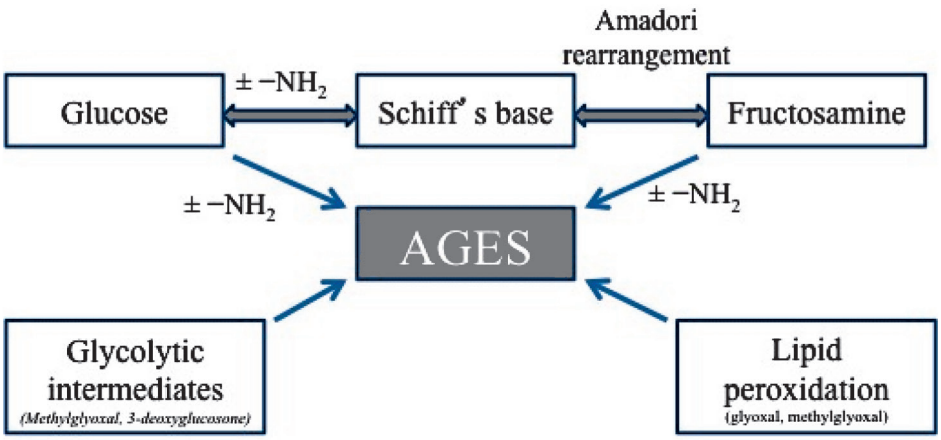
Inflammation may contribute to AGE formation. Activation of neutrophils, monocytes and macrophage result in release of myeloperoxidase and NADPH oxidase enzymes. These enzymes may react with amino acids to form AGE.[6]

Another cause of AGE formation or accumulation is decreased renal clearance of AGE precursors in renal insufficiency.[4] On top of this, renal insufficiency causes oxidative stress causing AGE formation.[4,7,8]

Finally, cigarette smoke and several food products are a source of AGE[9,10,11]. Some foods are extremely rich in AGE like for example crème brulée. Fortunately, only

10% of ingested AGE are indeed absorbed by the intestine.[10] An interesting detail: caramellisation of sugar is fact is nothing less than degrading sugar into AGE by heating.

Figure 1: Schematic view of the complex Maillard reaction and other pathways leading to the formation of AGE.



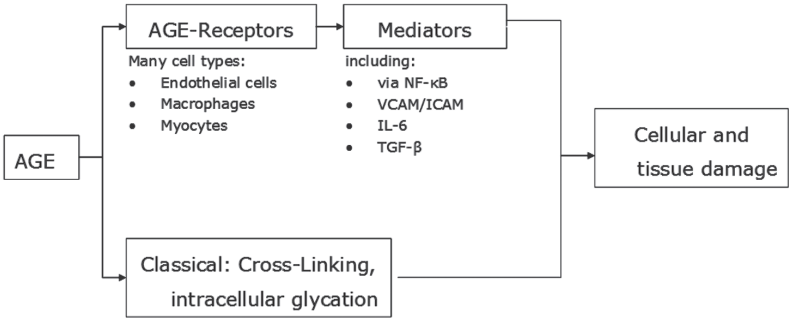
Accumulation of AGE in long lived tissues: a metabolic memory

In the process of AGE formation molecules are irreversibly changed and deformed. In tissues with a short life time, the deformed molecule is quickly degraded, limiting any negative effects. However, in long lived tissues AGE continue to accumulate.[12] Collagen in the dermis and vascular wall has a lifespan of 15-20 years while collagen in cartilage has an even longer lifetime and collagen in the crystalline lens of the eye even endures a lifetime. The deformation of long lived tissues by AGE is regarded as a normal process with ageing.[13] An increase of AGE accumulation is however seen in several diseases: diabetes mellitus (as a result of hyperglycemia),[14] renal failure (as a result of decreased excretion of AGE precursors and increased oxidative stress)[4,8] and chronic inflammatory diseases like rheumatoid arthritis and systemic lupus (as a result of activation of neutrophils, monocytes and macrophages).[15-17] The accelerated AGE accumulation seen in those conditions contributes to long term complications and mortality.[18-21] AGE accumulation in long lived tissues, therefore, seems to reflect a metabolic memory of endured glycemic, oxidative and carbonyl stress. It represents irreversible end organ damage.

The negative consequences of AGE accumulation

There are several reasons why AGE accumulation has deleterious effects. First of all, AGE formation changes the basic (tertiary) structure of the affected protein, lipid or DNA leading to dysfunction. Also, AGE can form crosslinks between molecules which interferes with normal function.[22,23] Crosslinking of AGE of vascular collagen/elastin contributes to arterial stiffness and crosslinking of skin collagen results in wrinkles.[24] Sometimes, AGE interfere with physiological degradation of the molecule. Adherence of AGE to cellular binding sites (especially mitochondrial) alters intracellular signalling and can ultimately lead to intracellular oxidative stress which causes a vicious cycle.[25] Finally, AGE can activate cell membrane receptors. The most notoriously known receptor to be activated by AGE is called the receptor for AGE (RAGE). This receptor is present on endothelial cells and several other cell types and leads to activation of several oxidative and inflammatory pathways.[26]

Figure 2: The mechanisms of damage by AGE. Crosslinking of proteins, intracellular glycation and activation of the receptor for advanced glycation end products (RAGE) all contribute to the deleterious effects of AGE accumulation.



Atherosclerosis and AGE

Increasing evidence shows that AGE play a major role in atherosclerotic disease, irrespective of the presence of diabetes mellitus and renal insufficiency.

How AGE can induce atherosclerosis is also explained by several mechanisms shown by in vitro studies. AGE interact with their receptors on endothelial cells, smooth muscle cells and macrophages. This induces focal adhesion molecules (VCAM-1), cytokine expression which may promote formation of atherosclerotic plaques.[27] Crosslinking of vascular collagen promotes vascular stiffness, hypertension and vessel

leakage.[24,28] AGE deposition may also attract monocytes to transmigrate into the vessel wall and subsequently release pro-atherogenic mediators.[29] Nitric oxide plays an important protective role in the vascular wall. AGE can (directly or via RAGE mediated mechanisms) reduce the release of nitric oxide or captivate nitric oxide.[30] AGE also trap LDL cholesterol in the subendothelium.[31] Glycoxidation of LDL particles induces loss of their affinity to bind to the LDL receptor, impairing hepatic clearance.[32] Also, uptake of LDL by scavenging receptors on macrophages and smooth muscle cells is promoted.[32] This all leads to increased LDL retention in the vessel wall and an increase in foam cells. Transformation to foam cells is also enhanced by RAGE receptor stimulation. Also, the cardioprotective function of HDL may be impaired by glycoxydation.[33,34] All these factors contribute to an accelerated progression of atherosclerosis as a result of AGE accumulation in the arterial vessel wall.

Besides these in vitro findings, evidence for a role of AGE in atherosclerosis also comes from animal studies. In rabbits, intravenous infusion of AGE and local application of AGE resulted in intimal thickening.[35] Conversely, a study in mice showed that treatment against AGE by either the soluble receptor for AGE (sRAGE), an AGE inhibitor (aminoguanidine) or AGE cross link breaker (ALT-711) resulted in an attenuation of atherosclerosis. A decrease in plaque area of 30-40% was observed.[36,37] Another experimental animal study showed that knocking out the receptor for AGE (RAGE) protected these animals against ischemic reperfusion injury.[38] Knocking out the receptor for AGE in diabetic apoE(-/-) mice reduced atherosclerotic plaque area to levels of non-diabetic mice. /-0.4%) to levels not significantly different from control apoE(-/-) mice.[39] Furthermore, a low AGE diet compared to high AGE diet resulted in a 50% fall in atherosclerotic burden in Apolipoprotein-E deficient mice.[40] NO-dependent vasorelaxation is significantly reduced by exposure to high glucose and the AGE methylglyoxal. However, glyoxalase-I transgenic animals (who, therefore, have a high glyoxalase-1 level that protects against methylglyoxal) have a normal NO-dependant vasorelaxation. This proves that AGE impair NO-dependent vasorelaxation and, therefore, interfere with normal endothelial function.[41]

Human data primarily show associations between AGE and atherosclerosis and vascular function. Serum levels of the AGE carboxymethyl-lysine were associated with increased aortic pulse wave velocity in adults.[42] Also, skin autofluorescence as a marker for AGE accumulation, is associated with arterial stiffness in patients with end-stage renal disease and in diabetes.[43,44] Patients with carotid disease have an elevated serum level of AGE. A positive association between intima media thickness (IMT) and serum levels of AGE was found in a population with renal insufficiency starting dialysis.[45] Also, AGE have been localized in atherosclerotic lesions, fatty streaks, lipid containing smooth muscle cells and macrophages.[46] This phenomenon was not only seen in patients with

diabetes, but also in normoglycemic patients.[47,48] Baumann et al. showed that the AGE N-epsilon-carboxymethyllysine (CML) is present in the subendothelial space of atherosclerotic human carotid artery material of normoglycemic subjects with a mean age of 50 year.[49] A clear correlation was found between tissue AGE concentration and severity of atherosclerotic lesions.

HOW TO MEASURE AGE ACCUMULATION?

Serum AGE levels

AGE are difficult to measure: it is not a single entity, but a group of structurally very diverse glycation end products. Different assays have been developed to assess serum levels of AGE with a known structure. Especially tandem mass spectrometry dependent methods, usually preceded by HPLC (high performance liquid chromatography) or UPLC, are now considered as the gold standard. Besides technical difficulties, the major problem is that serum levels of AGE simply do not necessarily reflect accumulation of AGE in long-lived tissue.[50,51] Also, assessment of a specific AGE does not necessarily reflect the whole group of AGE.

Tissue AGE accumulation

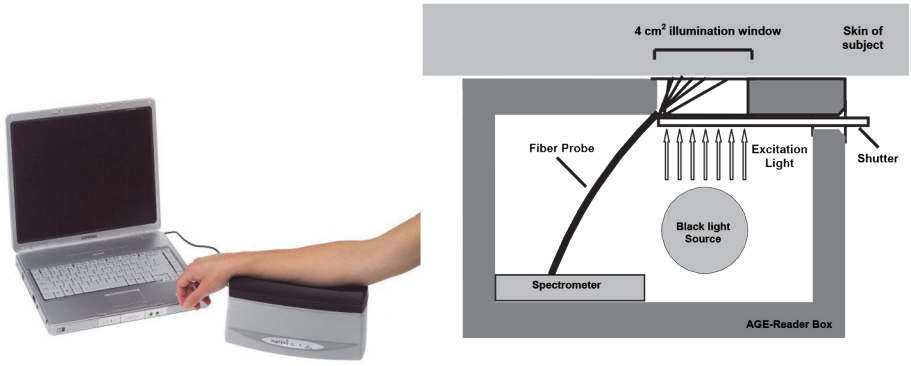
Tissue AGE accumulation can be assessed in skin biopsies, but the process is invasive and time consuming. Genuth et al showed that tissue AGE levels in skin biopsies predicted future progression of diabetic complications.[19] There is the pitfall of focusing on one specific AGE instead of the whole group. A method that gives a broader view of AGE accumulation is the protein cross-linking index in collagen rich biopsy material, but it has a low specificity. A different method, first introduced by Monnier et al in the 1980s, uses the fluorescent properties of some AGE and measures collagen linked fluorescence which gives an indirect quantitative measure of tissue AGE accumulation.[52] Unfortunately, having to take skin biopsies in order to measure tissue AGE accumulation is a practical barrier for routine use in medical practice.

The AGE Reader

A noninvasive method to estimate AGE accumulation in tissue became available in 2006: the AGE reader (Diagnoptics Technologies, Groningen, The Netherlands).[53] This technique uses the autofluorescent properties of some AGE and assesses accumulation of AGE in tissue through illumination of the skin (figure 3). The AGE Reader illuminates a skin surface of 4 cm^2 on the volar site of the underarm guarded against surrounding light. The excitation light source has a peak excitation of 370 nm (with a range of 350-420 nm).

This wavelength is in the UVA spectrum. Emission light in the wavelength range of 420-600 nm (fluorescence) and excitation light that is reflected by the skin with a wavelength range of 300-420 nm is measured with a spectrometer. Skin autofluorescence (SAF) is the ratio between the emission light (fluorescence) and the reflected excitation light. This ratio is multiplied by 100x and expressed as arbitrary units (AU). SAF has previously been validated by simultaneous measurements of SAF and contents of specific AGE assessments in skin biopsies. Although the fluorescent characteristics used by the AGE reader are not specific for fluorescent AGE, multiple validation studies have shown convincingly and consistently that SAF has a strong correlation with specific AGE content in skin biopsies. The correlation between skin AF with the fluorescent AGE pentosidine is very high: r=0.87. Surprisingly, not only fluorescent AGE (pentosidine) but also non fluorescent AGE (N-carboxymethyl-lysine (CML) and N-carboxyethyl-lysine (CEL)) in the skin biopsies showed great correlation with SAF. Skin AGE content explained the major part of the variance (up to 76%) in the SAF signal in a pooled analysis of three validation studies.[53,54] Therefore one can conclude that skin autofluorescence can be used as an indirect, noninvasive estimate of AGE accumulation in tissue.[53]

Figure 3: The AGE Reader illuminates a skin surface of 4 cm^2 on the volar site of the underarm guarded against surrounding light. The excitation light source has a peak excitation of 370 nm (range of 350-420nm). Emission light in the wavelength range of 420-600 nm (fluorescence) and excitation light reflected by the skin (wavelength 300-420 nm) is measured with a spectrometer. SAF is the ratio between the emission light (fluorescence) and the reflected excitation light.



A device comparable to the AGE reader, the SCOUT (developed by the US-based company Veralight Inc) has also been developed.[55]

Furthermore, a Fluototron device has been used in ophthalmology for measuring corneal or lens autofluorescence, which has been related to local AGE levels.[56]

AIMS OF THIS THESIS

This thesis has two major aims. After depicting the background of AGE and SAF, the first aim is to explore possible factors that interfere with the measurement of SAF. The second aim is, to investigate the relation between SAF and atherosclerosis in different vascular beds.

Part I presents an overview of the existing knowledge on advanced glycation end products (AGE), with chapter 2 focussing on their role in renal failure. This background information forms the starting point of this thesis.

Part II addresses different factors that may influence the measurement of SAF. In previous studies, SAF proved to be a predictor of mortality and complications in type 2 diabetes mellitus and renal failure. As the use of SAF is increasing, it is important to assess and review what factors might influence or disturb the measurement. Chapter 3 explores the influence of short- and medium term glycemic variation on SAF. The effect of AGE rich meals, postprandial rise in glucose levels, impaired glucose tolerance and HbA1c on SAF is studied. Chapter 4 investigates the influence of several dermal factors that could possibly disturb SAF measurements. Effects of several skin creams, hyperemia, vasoconstriction and hydration/moisture of the skin are evaluated.

Part III of this thesis focuses on SAF, as a measure of AGE accumulation, in patients with atherosclerotic disease in different vascular beds. Chapter 5 evaluates SAF in a small cohort of patients with carotid artery stenosis compared to healthy age matched controls. Chapter 6 addresses SAF in patients with peripheral artery disease (PAD). We present a case control study of 492 patients with PAD and 164 controls, matched for age and presence of diabetes mellitus. Cardiovascular risk factors and comorbidity (coronary artery disease, cerebrovascular disease, abdominal aortic aneurysm are assessed. Chapter 7 addresses the relation between SAF and diabetic complications in a secondary care, hospital-controlled group of patients with type 2 diabetes. We analysed 563 subjects with type 2 diabetes mellitus followed in 5 Dutch hospitals. It is a validation study to confirm previous findings in a single center primary care setting which showed that SAF is markedly increased in patients with diabetes mellitus type 2 and has a graded relation with diabetic complications.

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Chapter 2

Advanced glycation end products in renal failure: an overview

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J Ren Care 2008 Dec;34(4):207-212

ABSTRACT

The article aims to present an overview of the existing knowledge on advanced glycation end products (AGE). They are moieties that bind to proteins, but also lipids and nuclear acids. AGE are formed during glycation and oxidative stress. Accumulation of AGE occurs especially in diabetes and chronic renal failure and plays a major pathogenetic role. The deleterious effects of AGE result from cross linking of proteins and activation of the receptor for advanced glycation end products. AGE accumulation can be non-invasively assessed by the skin autofluorescence reader. In diabetics, skin autofluorescence predicts cardiac mortality and the occurrence of macro- and microvascular complications. In patients on haemodialysis, skin autofluorescence is highly elevated and predicts mortality. After renal transplantation AGE accumulation is lower than during haemodialysis, but still remains elevated and is a strong risk factor for chronic renal transplant dysfunction. Some of the potential methods to intervene with AGE accumulation are discussed in this article.

INTRODUCTION

Advanced glycation end products, also called AGE, play a major pathogenetic role in many age related diseases. They are also a cornerstone in the development of complications associated with diabetic nephropathy and chronic kidney disease (CKD). Here an overview is presented of the existing knowledge on AGE, focussing on their role in renal failure.

What are advanced glycation end products?

Advanced glycation end products (AGE) are glycated moieties that bind to proteins, but also lipids and nuclear acids. AGE are formed during glycation and also during oxidative stress. Glycation can result from several different mechanisms. Classically, advanced glycation end products are formed by a complex and sequential nonenzymatic reaction, called the "browning reaction" first described by Maillard in 1912. Hyperglycaemia and diabetes mellitus contribute to an enhanced formation of AGE by this Maillard reaction. During the process intermediate products, called Amadori products, are formed. A very well known Amadori product is the HbA1c, a glycated haemoglobin used to monitor diabetic regulation. AGE can also form during oxidative stress as a result of reactive carbonyl products.[1] For example, oxidative stress as a result of a myocardial infarction may cause a significant temporary rise in AGE (submitted data). Intake of many food products and smoke, containing AGE are the third source of AGE.[2,3] Fortunately, only 10% of ingested AGE are indeed absorbed by the intestine.[3]

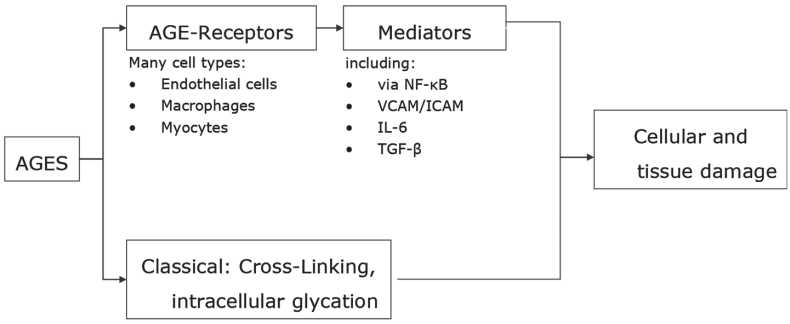
Accumulation of AGE

The linking of AGE to a protein can occur on any protein in the body. The resulting alteration is irreversible and chemically stable. Therefore, the lifetime of the AGE is determined by the lifetime of the protein they form on. Skin collagen, lens crystallins and cartilage proteins are very long-lived proteins and as a result AGE accumulation on these proteins continues throughout lifetime.[4] AGE linked to such long-lived proteins are therefore considered to be a long-term memory of both glycaemic stress (especially in diabetes) and oxidative stress. On short-lived proteins, the protein and its linked AGE product will be rapidly degraded to AGE peptides which are subsequently excreted by the kidney. The clearance of AGE modified peptides is highly effective with clearances ranging from 35 to 90 percent. In renal failure however, the excretion of AGE is impaired, resulting in enhanced accumulation of AGE.[5]

Mechanisms of damage by AGE

Tissue damage as a result of AGE formation may be the result of several mechanisms (figure 1). First of all, AGE formation leads to cross linking between proteins. This affects both the conformational structure and the function of proteins. These modifications of proteins occurs extracellularly as well as intracellularly.[6,7] Secondly, AGE bind to receptors, especially the receptor for advanced glycation end products (RAGE). Activation of this receptor induces intracellular transduction mechanism and cellular activation which has deleterious effects.[8,9] Many of the adverse effects of aging, diabetes and chronic renal failure can be attributed to accumulation of AGE. For example, wrinkling of the skin is the result of accumulation and crosslinking of AGE in collagen fibres of the skin. Atherosclerosis and vascular stiffening is associated with AGE accumulation in the vessel wall. AGE accumulation in the basement membrane of the glomeruli causes albuminuria and eventually chronic renal failure. Senile cataract and arthrosis are other manifestations of AGE accumulation in the lens and cartilage, respectively.

Figure 1: The mechanisms of damage by AGE. Crosslinking of proteins, intracellular glycation and activation of the receptor for advanced glycation end products (RAGE) all contribute to the deleterious effects of AGE accumulation.



Methods to measure AGE accumulation

AGE can be measured by biochemical and immunochemical assays. Also, AGE levels can be assessed using their characteristic fluorescent properties. On excitation at 370 nm, some AGE have a typical fluorescence spectrum at 440 nm. Serum levels of AGE do not necessarily reflect accumulation of AGE in tissue however.[10] Quantification of AGE accumulation in tissue is a more accurate measure of tissue damage and can be done by biopsy of tissue with subsequent determination of AGE levels. This is an invasive, complex

and expensive procedure. In 2004, a simple and non-invasive method of assessing AGE accumulation in skin using skin autofluorescence was first described (figure 2). Skin autofluorescence measured by the so-called autofluorescence reader correlates closely with AGE measurement by skin biopsy. Furthermore, the reproducibility of the skin autofluorescence measurement is good (Altmann error rate of 5.03%). A measurement takes less than one minute and reports the results immediately, making it a practical tool in daily patient care.[11]

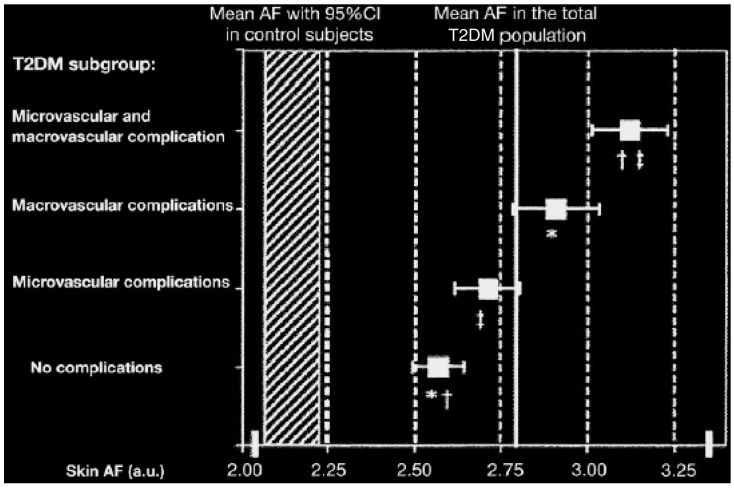
Figure 2: The autofluorescence reader. The forearm is positioned on the autofluorescence reader. The device measures the skin autofluorescence within one minute. The measured value of autofluorescence is reported immediately on the computer screen.

Diabetic nephropathy

Diabetes is the most prevalent cause of renal failure. This is illustrated by the fact that haemodialysis is the result of end stage diabetic nephropathy in approximately 20% of cases.[12] Patients with diabetes mellitus have a significantly higher AGE level than the normal population as a result of glycaemic stress. AGE have long been known to play a major role in the pathogenesis of diabetic macro- and microvascular complications, including diabetic nephropathy. The AGE levels correlate with the severity and duration of diabetes. Monnier showed in a cross-sectional study with 216 type 1 diabetics that AGE accumulation in the skin not only correlates with the severity and the duration of diabetes, but also with the presence of long-term diabetic complications. This association

was so strong, that skin AGE levels explained 19-36% of variance in the incidence of diabetic complications in intensively treated diabetics and 14-51% in conventionally treated diabetics.[13] Another large cross-sectional study on 973 well regulated type 2 diabetics showed that skin autofluorescence measured by the autofluorescence reader is significantly higher in type 2 diabetics with complications than those without complications.[14] Skin autofluorescence was highest in patients with both micro- and macrovascular complications (figure 3). In prospective studies, AGE measurement by skin autofluorescence also proved to be a strong predictor of cardiac mortality in diabetics.[15] AGE measurement may even add information to the standard estimation of cardiovascular prognosis by the UKPDS risk-engine (H. Lutgers, unpublished data, reported at the EASD 2007). Moreover, skin autofluorescence independently predicts the risk of developing microvascular complications like microalbuminuria and neuropathy. This has been proven in a 10 year follow-up study with 211 type 1 diabetics and recently in a 3 year follow-up study of the cohort of 973 type 2 diabetic patients mentioned earlier.[16,17]

Figure 3: Mean skin autofluorescence (AF) with 95% confidence intervals in different categories of complications in type 2 diabetics. The lined column represents the mean autofluorescence of the control group. The mean autofluorescence in the diabetic population is significantly higher than in the control population. Diabetics with no complications had significantly lower mean autofluorescence than diabetics with complications. Autofluorescence was highest in diabetics with both macro- and microvascular complications.

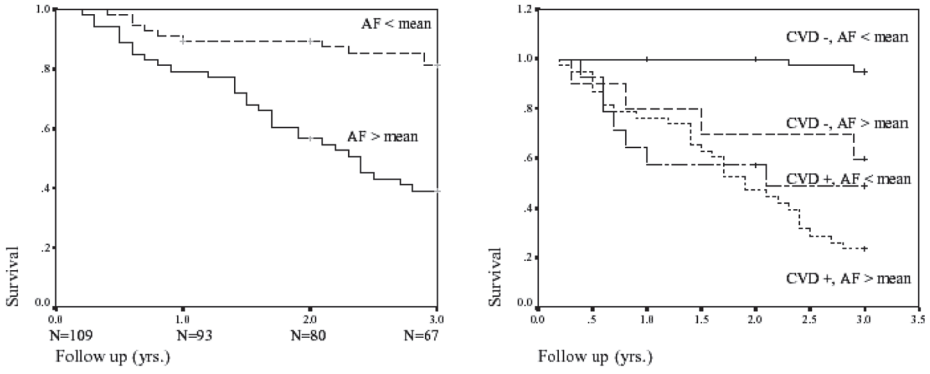


From: Skin autofluorescence as a noninvasive marker of vascular damage in patients with type 2 diabetes. Lutgers HL, Graaff R, Links TP, Ubink-Veltmaat LJ, Bilo HJ, Gans RO, Smit AJ. Diabetes Care. 2006 Dec;29(12):2654-9.

Chronic kidney disease

AGE levels are grossly elevated in chronic kidney disease and haemodialysis as a result of decreased clearance, resulting in an increased tissue accumulation. The accumulation of AGE in renal failure is even greater than in diabetes. Mortality is extremely high in patients on haemodialysis and approximates 10% annually.[18] High skin autofluorescence as a measure of AGE accumulation has been proven to be a predictor of both overall and cardiovascular mortality in haemodialysis patients[19] (figure 4). Peritoneal dialysis results in glycemic stress as a result of high glucose contents of the dialysis solution. AGE have been shown to accumulate in the peritoneum, contributing to peritoneal degeneration and eventually technique failure.[20] Strikingly, the use of peritoneal fluids low in glucose degradation products results in prolonged technique survival and (more importantly) also significant patient survival.[21] Peritoneal fluids with low to minimal glucose degradation products have therefore gained more widespread use in recent years. After renal transplantation, skin autofluorescence slowly falls and is lower than in patients on haemodialysis, but still remains markedly elevated in spite of a dramatic reduction in plasma AGE.[22] The cardiovascular morbidity and mortality also remain elevated after transplantation, presumably as a result of persisting AGE accumulation. Furthermore, high levels of skin autofluorescence increase the risk of chronic renal transplant dysfunction.[23]

Figure 4: Kaplan-Meier estimates of survival during follow-up with regard to overall mortality in patients on haemodialysis in relation to skin autofluorescence (AF) above and below mean values and the presence of cardiovascular disease (CVD) at baseline.



From: Skin autofluorescence, a measure of cumulative metabolic stress and advanced glycation end products, predicts mortality in hemodialysis patients. Meerwaldt R, Hartog JW, Graaff R, Huisman RJ, Links TP, den Hollander NC, Thorpe SR, Baynes JW, Navis G, Gans RO, Smit AJ. J Am Soc Nephrol. 2005 Dec;16(12):3687-93.

Therapeutic options

No drugs that focus on reducing AGE formation or breakdown of crosslinks have yet been introduced for clinical use. Several human studies are currently in progress however. The AGE formation inhibitor Benfotiamine and crosslink breaker Alagebrium are now being tested. Soluble RAGE analogues are also under development to scavenge circulating AGE. Angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB) are known to reduce macro- and microvascular complications of diabetes and also progression of renal failure irrespective of antihypertensive effects. Interestingly, these ACE inhibitors and ARB have been shown to reduce the formation of AGE.[24,25] Also, ACE inhibitors appear to up regulate the soluble receptor for AGE.[26]

Reducing the intake of AGE in food and smoke also is a possible target to lower AGE accumulation.[3] A diet low in AGE is unpalatable, however. This makes clinical use difficult, especially since patients with renal failure usually already have many dietary restrictions. Cessation of smoking, also a source of exogenous AGE, is also for other reasons obviously an important and longstanding goal in preventing cardiovascular events in both diabetes mellitus and renal failure.

More effective removal in AGE precursors by choosing specific dialysis methods or dialysis membranes is still under investigation. Daily haemodialysis improves AGE levels.[27] Vitamin E coated dialysis membranes also reduces AGE in patients on haemodialysis.[28] Very high flux polysulfone filters reduce predialysis serum levels of AGE.[29] Peritoneal dialysis solutions with a lower glucose content also reduce serum AGE as a result of a reduction of glycaemic stress.[30] Recent preliminary data show that skin autofluorescence is also lower in patients on low or no glucose containing dialysates. The same seems to hold for patients on high flux dialysates (unpublished data). This suggests that over time the lower serum AGE levels may also lower tissue AGE accumulation and therefore limit the resulting damage.

Renal transplantation is a most effective treatment for lowering AGE in patients with renal failure, although AGE accumulation in tissue remains high in comparison with the normal population.[22]

Conclusion

Advanced glycation end products accumulate in tissue during aging, diabetes and renal disease. AGE accumulation in the skin can be non-invasive and rapidly assessed by the autofluorescence reader. AGE accumulation in tissue has many deleterious effects and contributes largely to the development of long-term complications and high mortality in both diabetes and chronic renal failure. High levels of skin autofluorescence predict mortality and complications in diabetics and renal failure.

Conflict of interest

A.J. Smit is founder of DiagnOptics B.V., The Netherlands, manufacturer of the AGE-Reader, which has been used as the device for performing skin autofluorescence measurements discussed in this review.

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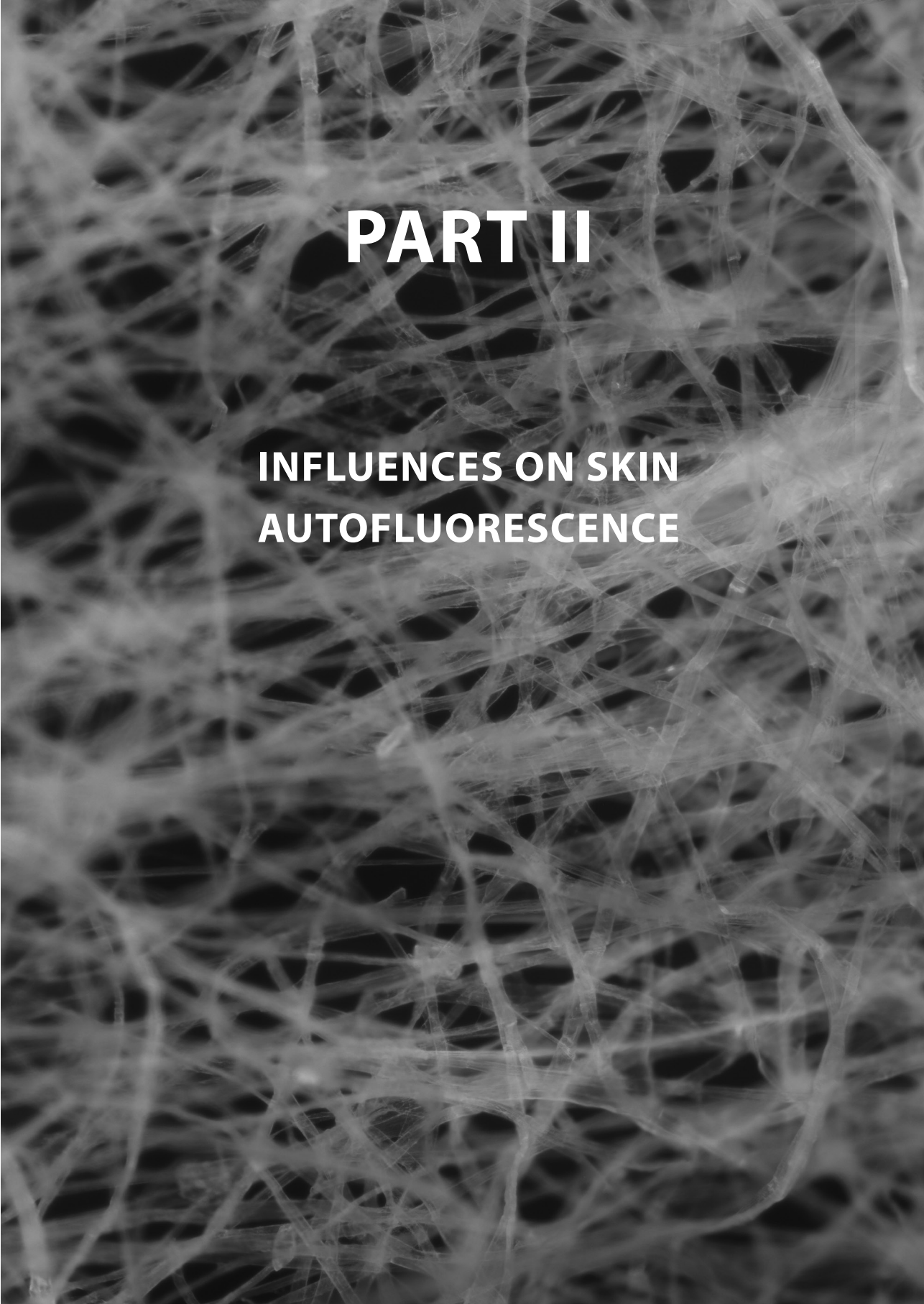

PART II

INFLUENCES ON SKIN AUTOFLUORESCENCE

Chapter 3

Skin autofluorescence and glycaemic variability

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Diabetes Technol Ther 2010 Jul;12(7):581-585

ABSTRACT

Background: Accumulation of advanced glycation end products (AGE) is accelerated during glycaemic and oxidative stress and is an important predictor of complications in diabetes mellitus (DM).

Study design: Here we both review and present original data on the relationship between skin autofluorescence (SAF), a non-invasive measure of AGE, and short- and intermediate term glycaemic variations.

Results: Acute changes in glucose levels during an oral glucose tolerance test in 56 persons with varying degrees of glucose tolerance did not influence SAF. AGE rich meals result in a transient postprandial rise in SAF of 10% 2-4 hours later. This could not be attributed to meal-induced glycaemic changes and is probably caused by the AGE content of the meal. In type 1 DM major intermediate term improvements of glycaemic control as depicted by multiple HbA1c measurements were associated with lower skin AGE levels. In a well controlled, stable type 2 DM cohort, only a weak correlation was found between SAF and HbA1c. In both studies skin AGE/SAF levels predicted diabetic complications with an accuracy superior to that of HbA1c. SAF has also been proposed as a new tool in diagnosing IGT and diabetes mellitus. It proved to be more sensitive than either fasting glucose or HbA1c.

Conclusions: SAF is not influenced by short term glycaemic variations. AGE rich meals may however cause a transient rise postprandially. There is a weak correlation between SAF or skin AGE and current or time-integrated HbA1c levels. SAF has strong added value in risk prediction of complications of diabetes and is a promising tool for early detection of diabetes and IGT.

Introduction

Advanced glycation end products (AGEs) play a major pathogenetic role in many age related diseases.[1] Especially in diabetes mellitus and renal failure, AGE accumulation is accelerated and causes long term complications and increased mortality.[2-6] Tissue and cellular damage as a result of AGE results primarily from AGE forming cross links between proteins, and from binding to and subsequent activation of cell membrane pro-inflammatory receptors including the receptor for advanced glycation end products (RAGE).[7]

Skin autofluorescence (SAF) has become a validated and widely accepted non-invasive method to assess tissue accumulation of AGE.[8] Not surprisingly, SAF proved to be a strong and independent predictor of complications in diabetes mellitus.[2] Also, it has prognostic value on top of the UKPDS risk score in predicting mortality in type 2 diabetics.[3,4]

AGEs, especially the accumulation in long lived tissues like skin collagen, have been proposed by several groups including the UKPDS group as a carrier of long term metabolic memory of glycaemic and oxidative stress.[9] Therefore, a correlation between SAF as a measure of tissue AGE and other parameters of glycaemic control might be expected. In this article we review and present original data on the relationship between glucose levels, HbA1c and SAF.

Skin autofluorescence and short term glycaemic variability

We studied the effects of short term glucose variations during a glucose tolerance test on SAF in 56 persons. Inclusion criteria were the presence of one or more risk factors for diabetes, namely obesity or other criteria for the metabolic syndrome, physical inactivity or an abnormal high glucose value or HbA1c in the past. Exclusion criteria were known diabetes, chronic kidney disease with a serum creatinine of >120 umoll/liter or an acute cardiovascular event in the previous 3 months. Subjects underwent an oral glucose tolerance test (OGGT) of 75 gram glucose dissolved in 120 ml of water. Glucose, HbA1c and SAF were determined at baseline. Furthermore, glucose and SAF were also measured two hours after ingestion of the 75 gram glucose. After the OGGT patients were classified into normal glucose tolerance, prediabetes and frank diabetes. Here, a normal glucose tolerance was defined as a fasting glucose of <5.6 mmol/l and a glucose of <7.8 mmol/l at the OGTT at 2 hours. Prediabetes was defined as a fasting glucose of 5.6-6.9 mmol/l or a glucose of 7.8-11.0 mmol/l at 2 hours of the OGGT. Diabetes was defined as a fasting glucose ≥7.0 or a glucose of >11.0 at 2 hours of the OGGT. Of the 56 subjects, 18 had a normal glucose tolerance, 25 had prediabetes and 13 diabetes. Baseline characteristics are shown in table I. No differences existed between

the different groups concerning sex, age, BMI, bloodpressure, tobacco use and serum creatinine. HbA1c at baseline was significantly different between the groups ($p<0.001$) with a higher HbA1c in the (pre)diabetes groups than in the normal glucose tolerance group as could be expected. Results concerning SAF are shown in table II. SAF at baseline appeared to be higher for IGT and diabetes than for normal glucose tolerance. SAF was 2.04±0.55 for normal glucose tolerance, 2.27±0.71 for IGT and 2.54±2.54 for diabetes. However, these differences were not statistically significant ($p=0.18$), probably as a result of the small number of subjects. Using ANOVA, the differences between the different glucose tolerance groups are evaluated. Glucose at baseline, at 2 hours and also the increase in glucose were significantly different in the three glucose tolerance groups, with increasing glucose values from normal glucose tolerance to diabetes. No increase of SAF at 2 hours after oral intake of 75 gram glucose was found compared to baseline, even though glucose rose significantly. SAF was 2.24±0.74 while fasting, and 2.22±0.65 at 2 hours. The changes in SAF were independent of baseline SAF and level of glucose tolerance or change in glucose levels.

In conclusion, SAF is not immediately influenced by a rise in serum glucose levels as measured during an OGGT.

Table I: Baseline characteristics in the total group and different glucose tolerance groups. Data are presented as mean ± SD or number of patients.

	Normal	Prediabetes	Diabetes	Total
N	18	25	13	56
Sex (males)	11	13	7	31
Age (years)	53,5±10,5	56,8±9,1	59,2±18,4	56,3±12,2
BMI (kg/m^2)	30,6±6,3	30,8±5,5	31,0±5,7	30,8±5,7
Diast BP (mmHg)	86±14	85±13	83±12	85±13
Syst BP (mmHg)	139±16	140±13	143±25	140±17
Current smoking	5	4	2	11
Creatinin (µmol/L)	78,1±26,3	82,7±16,6	76,5±21,1	79,7±20,9
HbA1C (%)	5,5 ± 0,3	6,0 ± 0,3	6,2±0,3	5,9±0,4

Table II: Results of OGTT on glucose and SAF in the total group and in the different glucose tolerance groups. Differences between the 3 glucose tolerance groups were evaluated by ANOVA and depicted by the p-value.

	Normal	Prediabetes	Diabetes	Total	p-value
Glucose 0 hr	5.1±0.26	6.0±0.46	6.93±0.93	5.9±0.87	<0.001
Glucose 2 hr	5.34±1.11	7.2±1.92	11.2±3.19	7.5±3.0	< 0.001
Δ Glucose	0.08±1.21	1.19±2.04	4.3±3.6	1.56±2.77	< 0.001
SAF 0 hr	2.04±0.55	2.27±0.71	2.54±0.96	2.25±0.74	0.18
SAF 2 hr	1.99±0.54	2.28±0.58	2.43±0.90	2.22±0.67	0.17
Δ SAF	0.04±1.65	0.01±0.25	0.11±0.18	0.033±0.21	0.25

The influence of an AGE-rich meal on skin autofluorescence

Food consumption may influence the accumulation of advanced glycation end products and thus SAF by several mechanisms. First of all, intake of food can result in glycaemic stress promoting the formation of AGE, especially when consisting of fast-acting carbohydrates. Over prolonged periods this will enhance the tissue accumulation of AGE. Secondly, food is an exogenous source of AGE with broiled and fried food having markedly high AGE content.[10,11] An AGE-rich meal might therefore influence the measurement of SAF, even when the short-acting glucose load has no effects on SAF, as discussed above. Stirban et al investigated the effect of a 580 kCal meal reported to have a intermediate AGE content of 8519 kU on SAF.[12] Actually, both the caloric and AGE content is substantial. In this study, SAF was measured in a fasting state, as well as 2 and 4 hours postprandially in 21 Caucasian subjects of which 10 were healthy and 11 had diabetes mellitus. A statistically significant increase of 10% in SAF was found 2 hours postprandially from 1.97±0.62 AU to 2.17±0.62 U. In the diabetic subjects, SAF increased 11.6% and glucose levels rose significantly from 7.55 to 8.55 mmol/l at 2 hours. In healthy subjects, there was an 8.7 % increase in SAF at 2 hours while glucose did not significantly rise (4.3 to 4.6). Individuals with diabetes had a slightly higher increase in SAF than the healthy individuals, 11.6 versus 8.7%. At 4 hours SAF had still not recovered and remained significantly elevated compared to baseline: 2.16±0.72 ($p<0.01$). We did a similar study with a breakfast of 2 toasted slices of bread and cheese and 220 ml of caramelized pudding which has an estimated AGE content of 2700 kU. The AGE content was estimated by the same method as Stirban et al by using lists with the specific AGE contents of different foods provided by Goldberg et al.[13] In 9 healthy subjects (18-20 years; 8 of the 9 subjects female) the SAF was measured before and at 1, 2 and 3 hours after the meal. At baseline SAF was 2.05±0.32. At 1 and 2 hours no rise in SAF was

observed with a SAF of 2.00±0.26 and 1.99±0.34 respectively. At 3 hours postprandially, however, the SAF was 9 % higher: 2.22±0.27 (p=0.038 in paired t-test). Results of both studies are presented in table III.

Table III: Results of an AGE rich meal on SAF. SAF is presented with mean ± SD. The statistical significance of the difference in SAF before and after the meal is depicted by the p-value.

	AGE content	N=	SAF 0 hr	SAF postprandial	Δ SAF	p-value
Stirban	8518 kU	21	1.97±0.62	2.17±0.62	0.20	<0.01
Own results	2700 kU	9	2.05±0.32	2.22±0.27	0.15	0.038

In conclusion, meals rich in AGEs may result in a postprandial rise of SAF of maximally 10%. Considering the lack of effect of glucose, described above, the meal-induced rise in SAF is probably due to the AGE content of the food. We therefore recommend not to measure skin autofluorescence after an AGE rich meal. The effect of meals with a normal to low AGE content are as yet unknown. For more information concerning the AGE content of different foods we refer to the available lists generated by Goldberg et al.[13] In general, unprocessed fruits, non-toasted bread and liquids do not contain a high amount of AGEs. Clearly, a fasting state would abolish all potential influences from food consumption. If these meal induced effects would be eliminated, the predictive value of SAF would even surpass the earlier reported values.

Skin autofluorescence and intermediate term glycaemic control measured by HbA1c

While SAF represents a long term memory of glycaemic and oxidative stress, HbA1c represents a glycaemic memory over a period of 3 months. HbA1c is considered an early glycation product or Amadori product. In monitoring glycaemic control in diabetic patients, HbA1C is normally measured repeatedly over time. A correlation between HbA1c as an early glycation end product and SAF as a marker of tissue accumulation of advanced glycation end products can be expected. Several researchers indeed analysed the relationship between HbA1c values over time and AGE accumulation in tissue measured either directly in skin biopsies or non-invasively as SAF.[14,15]

Gerrits et al examined 452 patients with type 2 diabetes in a primary care setting who were well controlled (mean HbA1c 7%).[15] She found that after 3.3 years of follow-up the accumulation of AGEs in skin measured as the rise in SAF at follow-up, had a weak, but significant correlation with several integrated assessments of HbA1c (variance of

HbA1c, mean HbA1c, maximum HbA1c, and HbA1c at baseline). Regression coefficients were <0.1; p≤0.025 (with addition of baseline SAF: overall adjusted R^2 ~0.45; p<0.001).[15] The major determinants of SAF at follow-up after 3 years were baseline SAF and age. Duration of diabetes and smoking were not, and renal function was of marginal predictive value.

In a subpopulation of the Diabetes Control and Complications Trial (DCCT) skin biopsies of 216 patients with type 1 diabetes were examined for the presence of various AGEs.[14,16] The biopsies were performed near the end of the trial. Of these subjects, 122 had been treated with intensive treatment and 94 had been treated conventionally during the previous 5 years. A strong positive relation between skin AGEs and age and duration of diabetes was found. Five years of intensive insulin therapy resulted in 24% lower HbA1c levels (7.1% in the intensive treatment group versus 9.3% in the conventional treatment group). Moreover, AGE's measured in the skin biopsies were lower after intensive insulin treatment. In the intensive treatment group CML and pentosidine were 9-13% and 9% lower respectively compared to the conventional treatment group. Cross linking of skin collagen was lower in the intensive treatment group resulting in a 24% higher acid soluble collagen and a 50% higher pepsin soluble collagen. The relationship between pentosidine and CML with different integrated HbA1c markers (mean HbA1c up to biopsy, mean HbA1c over the past year, HbA1c nearest to biopsy and screening HbA1c) was assessed by performing univariate regression analysis. The relationship was again weak, but significant with R^2 9.6 and 13.8 for pentosidine and CML respectively with HbA1c nearest to biopsy; 8.9 and 16.1 for mean HbA1c up to biopsy.

In conclusion, both Gerrits et al and Monnier et al found a weak relation between HbA1c and AGE's in skin measured either as SAF or directly in skin biopsies. Monnier et al found HbA1c and skin AGE levels similarly lower in the group with 5 years of intensive treatment than in the conventional treatment group. The better glycaemic control no doubt resulted in a drop in glycaemic stress and (to a lesser extent) oxidative stress.

The issue of predicting diabetic complications by either HbA1c or AGEs measured in skin biopsies was also addressed by Monnier et al and Genuth et al.[14,16] Monnier et al cross sectionally found that all AGE's measured in the skin biopsies were significantly associated with diabetic complications like retinopathy, nephropathy and neuropathy. After another 5 years of follow-up in this subpopulation of the DCCT, Genuth found that the combination of furosine (glycated collagen) and the AGE carboxymethyllysine (CML) predicted the risk of progression of retinopathy and nephropathy over the 10 year follow up period. The predictive value of skin AGEs remained after adjustment for HbA1c. Moreover, the predictive value of HbA1c completely disappeared after correction for furosine and CML. They concluded that accumulation of AGEs in tissue appears to be a major contributor in the development of diabetic complications and explains the

phenomenon of metabolic memory. In another study, Lutgers et al found that AGEs non-invasively measured as SAF proved to have predictive value far surpassing the value of HbA1c as established by several studies.[2-4,14,17] The SAF added information to the classical UKPDS in which HbA1c, duration of diabetes and the classical risk factors for atherosclerosis are included.[4]

Value of skin autofluorescence compared to IFG and HbA1c in diagnosing diabetes mellitus and IGT

Maynard et al evaluated the value of SAF in detecting undiagnosed diabetes mellitus or impaired glucose tolerance (IGT) in a naïve population.[18] They used an oral glucose tolerance test as the golden standard. A two hour OGTT of >7.8 mmol/l was used to define IGT and values of >11.1 mmol/l defined diabetes mellitus. From a total of 351 participants impaired glucose tolerance was found in 55 subjects and diabetes in 29 subjects. A fasting glucose of >5.5 mmol/l had a sensitivity of 58% and a specificity of 77%. The authors chose to examine the sensitivity of HbA1c and SAF at this particular specificity of 77%. At the specificity of 77%, HbA1c>5.8 mmol/l did better with a sensitivity of 64%. SAF however was superior to both fasting glucose and HbA1c with a sensitivity of 75% at the predefined specificity of 77% with was a statistically significant improvement over blood tests ($p<0.05$). SAF, therefore, diagnosed 29% more subjects with abnormal glucose tolerance than fasting glucose and 17% more than HbA1c. The authors concluded that as 30 % of diabetics are undiagnosed the use of SAF might prove a powerful tool for early detection of abnormal glucose regulation and prevention of resulting damage.

Preliminary, as yet unpublished results in a group of 57 subjects at risk for IGT or diabetes confirmed the superiority of SAF above fasting glucose and HbA1c in classifying subjects in categories of normal glucose tolerance versus IGT and diabetes. Using SAF as part of a simple decision tree led to misclassification of 4 out of 57 persons, while a fasting glucose >6 mmol/l would have led to misclassification of 17 subjects and using glycated hemoglobin (HbA1c) values of >6% to misclassification of 14 subjects. Validation studies in other and larger cohorts are ongoing (Smit A.J., preliminary results presented at ATTD 2009, Athens).

Conclusion

SAF is not significantly influenced by short term glycaemic variations as measured during an OGTT. An AGE rich meal may result in a postprandial rise after 2-4 hours in SAF of approximately 10%. Intermediate term glycaemic variations as measured by multiple HbA1c measurements (over time) are weakly related to SAF both in type 1 and type 2 DM, but dramatic improvements in HbA1c over years may be associated

with a lower level of AGE in skin. Moreover, skin AGE and SAF are strong predictors of diabetic complications and mortality with an accuracy far exceeding that of HbA1c and even that of most constituents of the UKPDS risk score. SAF is also a promising tool in diagnosing IGT and diabetes mellitus and proved to be more sensitive than either fasting glucose or HbA1c.

Conflict of interest

A.J. Smit and R.Graaff are founder and stockholder of DiagnOptics B.V., The Netherlands, manufacturer of the AGE-Reader, which has been used as the device for performing skin autofluorescence measurements discussed in this study.

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Chapter 4

Dermal factors influencing measurement of skin autofluorescence

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ABSTRACT

Background: Skin autofluorescence (SAF) is a non-invasive marker of accumulation of advanced glycation endproducts. It predicts cardiovascular complications and mortality in diabetes and renal failure. We assessed the influence of potential common confounders in SAF measurement, by determining the effects of endogenous and exogenous local dermal changes by body creams, hyperemia, vasoconstriction and hydration.

Methods: SAF was measured before and after local administration of body lotion, day cream, sun screen or self browning cream and after attempts to remove these effects with alcohol swabs and washing. SAF was measured before and during three hyperemia maneuvers, vasoconstriction and on a dry and wet skin.

Results: The body lotion increased SAF by 18%. Day cream, sun screen and self browning cream gave an increase >100%. Except for body lotion, subsequent cleaning with alcohol swabs and washing with soap did not return SAF to baseline values. The effect of self browning cream persisted for 2 weeks and of sun screen for 4 days. Hyperemia caused by either a hot bath, capsicum cream or post occlusive reactive hyperemia gave a decrease in SAF of respectively 18, 22 and 2.3%. Vasoconstriction caused by immersing the arm in cold water gave a 10% increase. Hydration state did not influence SAF.

Conclusion: Measurement of SAF is strongly affected by several skin creams. This effect was often not fully corrected by alcohol swabs and washing with soap and may persist for many days. Marked hyperemia and vasoconstriction also influence SAF. We advise to avoid these potential error sources.

INTRODUCTION

Advanced glycation end products (AGEs) accumulate in tissue over a lifetime, which is regarded as a process of normal ageing.[1] It is well known that AGE accumulation is accelerated in diabetes mellitus and renal failure and contributes to long term complications and mortality.[2-6] AGEs play a major pathogenetic role in many age-related diseases as a result of cross link formation and activation of cell membrane receptors including the receptor for AGE (RAGE) which leads to activation of several oxidative and inflammatory pathways.[7-9]

Accumulation of AGEs in tissue can be assessed noninvasively with a technique named skin autofluorescence (SAF), which uses UV light for the excitation of fluorophores in the skin. This technique has previously been validated by simultaneous measurements of SAF and assessments of specific AGE from skin biopsies of the dermal layer at the same measuring site.[10] We earlier reported increased SAF in several groups of patients with increased AGEs formation such as diabetes mellitus, decreased clearance of AGEs such as renal failure and overt atherosclerotic disease such as patients with stable coronary artery disease.[5,11,12] Moreover, SAF proved to be a powerful predictor of complications and mortality in diabetes and renal failure.[2,3,5]

Clinical application of SAF to estimate risk in diabetes mellitus and renal failure is gaining ground. For adequate application of SAF it is important to know what factors might influence or disturb the measurement. The effect of excitation wavelength and skin color in general have been previously studied and reported.[13,14] Also, the influence of an AGE rich meal resulting in a temporary postprandial rise of 10% has been reported by Stirban et al.[15] The present study addresses the influence on SAF measurements of potential common confounders inducing local exogenous and endogenous dermal changes. First, the influences of several skin sun protecting and tanning creams on SAF are evaluated. Second, we measured the effects of hyperemia and vasoconstriction. Third, the effect of wetness of the skin was addressed.

MATERIALS AND METHODS

Subjects

Healthy volunteers from the normal population were recruited to participate. Informed consent was obtained. Inclusion criteria were Caucasian race and an age between 18 and 65 years. Exclusion criteria were the use of medication, a prior medical history or any current medical problem.

Assessment of SAF

To assess tissue AGE accumulation, SAF was measured with the AGE Reader™ (DiagnOptics Technologies BV, Groningen, the Netherlands). The AGE Reader™ is a desk-top device that uses the characteristic fluorescent properties of some AGEs to estimate the level of AGE accumulation in the skin. Technical details of this non-invasive device concerning the optical technique have been described more extensively elsewhere.[14,10] In short, the AGE Reader illuminates a skin surface of 4 cm^2 guarded against surrounding light with an excitation light source with a peak excitation of 370 nm. This wavelength is in the UVA spectrum. Emission light in the wavelength range of 420-600 nm (fluorescence) and excitation light that is reflected by the skin with a wavelength range of 300-420 nm from the skin is measured with a spectrometer. SAF was determined from the ratio between the emission light and the reflected excitation light, using the AGE Reader software version 2.2. In the current series of experiments, the forearm was positioned on top of the device in the usual manner as described by the manufacturer. For each SAF value, a series of three consecutive measurements was carried out, which took less than a minute time. The mean of these three consecutive measurements was calculated and used in the analyses.

Study protocol

First, several representatives of dermal creams were tested. Body lotion (Etos®), facial day cream (Etos®), water resistant sun screen with a UVA/UVB protective value of 50 SPF (Etos®) and self browning cream containing dihydroxyaceton (DHA) manufactured by Vichy® were evaluated. Specifications of the contents of the creams are provided in the appendix. Beforehand, we tested the autofluorescent and light absorbing properties of the creams itself on a calibration material which has zero autofluorescence and a practically 90-100% reflectance. Then, at baseline, a standard triple measurement of SAF was performed before application of the cream on the lower arm of the study subjects. SAF was again measured 5-10 minutes after application of the cream permitting time for the cream to fully draw into the skin. After this, SAF was measured after rubbing with an alcohol swipe and subsequently after washing the skin with water and soap in an attempt to clear the skin of the applied cream. Subsequent alcohol swipes or washing were not done in the case of browning cream. Finally, we assessed the natural time for the effect of sunscreen and DHA cream to wear off. We chose these 2 creams as they proved in previous experience and in the initial steps to have the most potent and persistent effects on SAF. The cream was applied and subsequently SAF was measured daily with subjects performing their normal daily routine.

Second, we investigated the effect of hyperaemia and vasoconstriction. Hyperaemia was elicited in several ways. First, by immersing the arm in warm water of 42 degrees

for 4 minutes. SAF was measured directly after drying the skin with a towel. Second, by applying a tourniquet for 4 minutes and measuring SAF during the post-occlusive reactive hyperaemia (PORH)/reperfusion phase 2 minutes after releasing the tourniquet. Third, by applying capsicum cream locally on the measurement site for 4 minutes, which results in an outspoken local vasodilatation of the skin within several minutes of application. SAF was measured after removal of the cream. Intrinsic autofluorescence and absorbance effects of capsicum cream are shown below. Vasoconstriction was induced by immersing the arm in cold water of 12 degrees for 4 minutes. After drying the arm with a towel, skin AF was immediately measured.

Last, the influence of local dermal wetness was addressed by measuring SAF before and directly after application of a wet cotton gauze for 5 minutes. After removal of the gauze the skin was not dried before SAF measurement.

Statistical analysis

Data were gathered in a database (SPSS 15-0, SPSS Inc, Chicago, Illinois, USA). Normal distribution of the variables was tested by Kolmorgorov-Smirnov tests. Descriptive statistics are, therefore, presented as mean with standard deviation in case of normal distribution, otherwise as median with interquartile range or as number of patients. A paired student t- test was used for normally distributed parameters and a paired Mann Whitney U test was used for parameters with a skewed distribution. We performed a power analysis and determined that 11 subjects were needed to detect a 5% difference in SAF (β=0.8, α=0.05).

RESULTS

Subject characteristics

We gathered 39 study subjects. All subjects were Caucasian, healthy and used no medication as defined by the inclusion and exclusion criteria. The median age was 31 years. Twenty subjects (51%) were male. Eight subjects (20%) were current smokers. Mean BMI was 23.4 kg/m^2. Mean blood pressure was 124/76 mmHg. At baseline median an SAF was 1.69±0.33 which is in agreement with the expected SAF of 1.69 for this mean age,[16] and mean skin UV reflectance was 17%±4.9%,.

Effect of different body creams

The intrinsic fluorescent and reflectance properties of the different creams as tested in vitro against the white standard are presented in table I. There were major differences in both (auto)fluorescence and reflectance between the creams. While body lotion

appeared to have negligible fluoresent properties, daycream and sun screen were highly fluorescent. Day cream and sun screen had very high absorbent properties resulting in a very low reflectance levels of 1.5% and 2.0%, respectively. Self browning cream and capsicum cream showed moderate autofluorescence and little UV absorption.

The effects of different body creams on lower arm SAF measurements are shown in table II. Body lotion gave an 18% increase in SAF. Facial day cream and sun screen, however, gave a 139 and 111% increase of SAF, respectively. Self browning body cream containing DHA even gave a tripling in SAF. This effect was accompanied by a marked: drop in reflectance for both facial day cream, sunscreen and self browning cream. For these 3 creams reflectance was lowered from 17% to approximately 3%.

Table I: Measurement of intrinsic fluorescence and reflectance of creams applied on calibration material with zero autofluorescence and 90-100 % reflectance. Data are denoted as mean (±SD)

	N	Autofluorescence (AU)	Reflectance (%)
Body lotion	2	0.076 (0.034)	79.5 (0.71)
Day cream	4	10.85 (1.32)	1.53 (0.096)
Sun screen	3	3.13 (1.00)	2.03 (0.40)
Self browning cream	4	0.90 (0.091)	59.5 (1.00)
Capsicum cream	3	1.03 (0.23)	56.7 (3.2)

Table II: Effect of the different creams on SAF (standard deviation).

		SAF (AU)			Reflectance (%)		
	N	Baseline	Cream	% increase	Baseline	Cream	% decrease
Body lotion	14	1.81 (0.25)	2.14 (0.40)	18*	16 (5)	14 (5)	12.5*
Day cream	8	1.86 (0.41)	4.45 (0.93)	139*	17 (5)	3 (1)	82.4*
Sun screen	10	1.61 (0.21)	3.40 (1.16)	111*	17 (6)	2 (1)	88.2*
DHA cream	3	1.95 (0.45)	7.77 (1.63)	298*	16 (6)	8 (3)	50*

* denotes statistical significance with a p-value of <0.001

The effect of cleaning the skin by rubbing with an alcohol swab and additional rinsing with water and soap are presented in figure 1. It proved to be relatively easy to eliminate the effects of body lotion on SAF: the effect on SAF was largely reversed by an alcohol swab. After an additional washing, no difference with the baseline SAF level was found. Alcohol swabs only marginally reversed the increase in SAF induced by the day cream

and sunscreen. Even additional careful washing of the arm with water and soap did not result in return of SAF to baseline values. With day cream, alcohol swabs gave a 33% fall of SAF towards baseline levels, and another 17% by washing. After the alcohol swab and washing SAF was still elevated compared to baseline: 2.47±0.64 versus 1.86±0.41 (p=0.002). Sunscreen proved to be the cream with the most persistent effects on SAF. Here, alcohol swabs gave only a 13% fall of SAF towards baseline levels, and additional washing another 19%. After the alcohol swab and washing SAF was still elevated from baseline: 2.42±0.32 vs. 1.61±0.21 (p<0.000). Reflectance also remained significantly lowered in spite of alcohol swaps and washing for both day cream and sun screen. We assessed the natural wear-off effect of sunscreen and self browning cream in 3 subjects to provide an indication. It took approximately 4 days with sunscreen, and 2 weeks with the self browning cream before SAF had returned to baseline levels (figure 2).

Figure 1: Effects of cream, alcohol swab and washing.

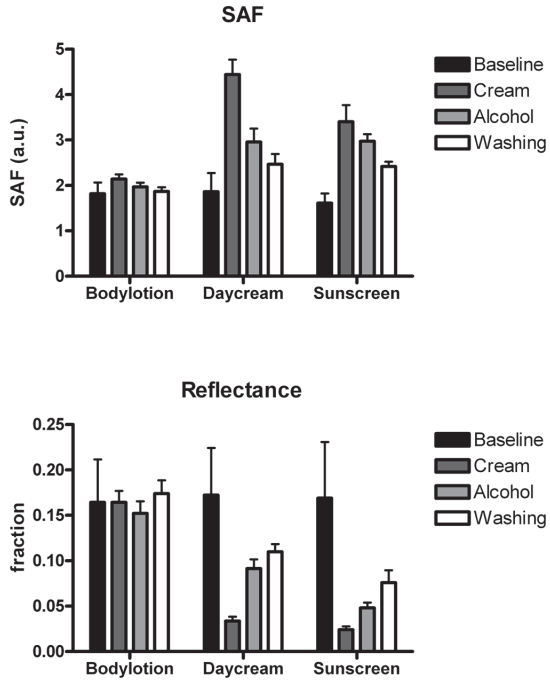
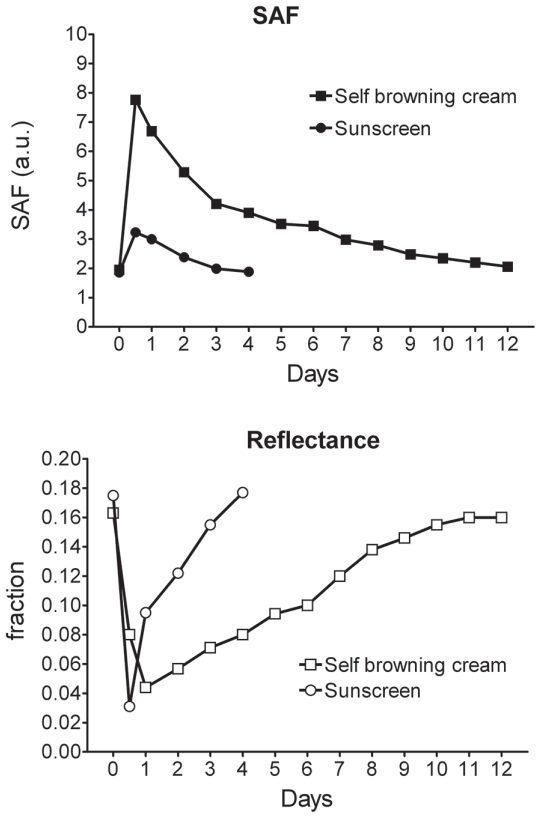


Figure 2: Wear off effect of self browning cream and sunscreen.



Effect of vasodilatation and vasoconstriction

The effect of vasodilation on SAF was tested by a hot bath, capsicum cream and reperfusion after release of a tourniquet. Results are shown in table III. Capsicum cream and a hot bath resulted in an outspoken visual hyperaemia, while reperfusion did not. SAF was lowered by 22% for capsicum cream and 18% after a hot bath. Reperfusion only resulted in a non-significant drop of 2.3% in SAF. During all hyperaemia manoeuvres, UV reflectance was not affected. Vasoconstriction caused by a cold bath caused a 10% higher SAF and a decrease in reflectance from 16.8 to 14.6 % (p=0.009).

Table III. Effect of hyperaemia and vasoconstriction on SAF (standard deviation). The p-value represents statistical difference from baseline with * denoting statistical significance.

	N	Baseline	Hyperaemia/ vasoconstriction	% difference	p
Cold bath	10	1.73 (0.33)	1.90 (0.38)	+10	0.026*
Hot bath	10	1.73 (0.33)	1.41 (0.49)	- 18	0.002*
Capsicum cream	12	1.76 (0.38)	1.37 (0.27)	- 22	0.002*
Reperfusion	18	1.71 (0.36)	1.67 (0.33)	- 2.3	0.11

Effect of hydration state of the skin

We measured SAF on a normal dry skin and subsequently on a wet skin after 5 minutes of application of a wet cotton gauze in 16 subjects. There was no difference in SAF between a dry and wet skin. On the dry skin SAF was 1.99 (±0.44) and on the wet skin 2.02 (±0.50), p=0.57. Reflectance also was not different: 17.9 versus 18.3%, p=0.51.

DISCUSSION

Our study aims to clarify pitfalls in the measurement of SAF. We show that both the application of several creams as well as extreme vasodilatation and vasoconstriction at the measurement site may have a major effect on SAF. Creams and extreme vasoconstriction can result in falsely high SAF whereas vasodilatation causes a lower value. This has to be taken into consideration when a SAF measurement is performed.

Creams

The increase in SAF after application of creams can be attributed to several different mechanisms. First of all, a cream may have fluorescent properties of its own as shown in table I. Secondly, some creams like day cream and sunscreen are designed to absorb UVA and UVB radiation to protect the skin against the long term harmful effects on skin photo-aging and the development of skin cancer. SAF is the ratio between the emission light (autofluorescence) and the reflected excitation light (with a peak wavelength at 370 nm in the UVA spectrum). Creams like sunscreen and day cream lead to a severe drop in the reflected excitation light, as shown by the lowering of reflectance. Because autofluorescence occurs by definition at higher wavelengths and, thus, is less influenced, the SAF as a ratio between the two would rise. Similarly, creams that show absorbion of the excitation light will cause a lower SAF. Third, creams may cause changes in the skin itself. As for self browning cream DHA binds to the stratum corneum leading to

chemical changes. This leads to a brown discoloration of the skin and an increased absorption of UVA light. As the self browning cream itself did not show marked intrinsic autofluorescent or absorbent properties, the large effects on SAF are probably explained by these chemical changes in the stratum corneum with resulting fluorescent and absorbent properties. Creams influencing capillary blood flow and hydration state may also result in dermal changes that affect SAF. In conclusion, creams may directly or indirectly influence SAF measurements.

We also showed that the effect of creams on SAF is not completely reversed by alcohol swaps or washing with water and soap. Besides that, it takes 4 days for sunscreen effects on SAF to naturally wear off, and even 2 weeks for self browning cream. The water resistant sunscreen we used was designed to not be easily washed off, but it is surprising to see that the effect persisted for 4 days. Self browning cream containing DHA chemically binds to the stratum corneum. The long delay of SAF to return to baseline levels is therefore, mainly determined by the turnover or rather wear off time of the stratum.

Blood flow

Local skin blood flow also influences SAF measurement. This phenomenon can be understood by the fact that haemoglobin has absorbent properties over a broad range in both the excitation and emission parts of the autofluorescent spectrum used during SAF measurements. As an increase in the amount of dermal blood causes lower values of SAF, we may conclude that more of this emission light (fluorescence) is absorbed compared to the absorbance of reflected excitation light. This can also be observed by the virtually unchanged reflected excitation light. Vasoconstriction leads to the reverse effect, in other words a higher SAF value. In our study, the effects were seen only when extreme vasodilatation was induced. Capsicum cream and the warm bath both led to a clearly visible hyperaemia and gave 22% and 18% decrease in SAF. Reperfusion after applying a tourniquet only led to a 2% decrease which was not significant. In this situation, there also was no visible hyperaemia. One must keep in mind that the AGE Reader only penetrates the skin to a depth of 0.1-0.2 mm and, therefore, only the effect of the most superficial capillaries are measured, mainly nutrient skin capillaries of the upper and lower dermal plexus.[17] This may explain the relatively modest effect of skin blood flow on SAF. With the post occlusive reactive hyperemia procedure, the resulting ischemia may have influenced the NAD/NADH balance. NADH has autofluorescent properties in the same wavelength as AGE's.[18] Surprisingly, during and after ischemia SAF was hardly influenced. This may be due to the fact that NADH fluorescence is found more in the epidermis where oxygen is provided by direct diffusion from the air, and therefore less influenced by arterial occlusion caused by a tourniquet. We, therefore,

are inclined to believe that NADH hardly influences SAF measurement. Vasoconstriction caused by a cold bath of 12 degrees led to a 10% increase in SAF. We conclude that only extreme vasodilatation and vasoconstriction significantly affect SAF.

Limitations

We are aware that we only examined a limited number of creams. A list of creams without effects on SAF cannot be provided. Results in the tested creams are, however, very marked and lead to the conclusion that caution in SAF measurements after the local use of any cream is necessary, especially for sun protecting creams or skin tanners. A low reflectance level in a normal white Caucasian skin should raise the suspicion of the recent use of such a cream. The manual provided by the manufacturer of the AGE Reader, therefore, advises not to measure SAF after recent use of skin creams. Perhaps some other spectral characteristics other than the reflectance level may also become helpful in detection of previous use of cosmetic or other preparations affecting a SAF measurement.

Reperfusion of the arm after application of a tourniquet did not lead to a significant decrease, while a warm bath and capsicum cream did. How to explain this discrepancy? Maybe reperfusion did not lead to enough vasodilatation to cause an effect. Also, measurements were taken 2 minutes after release of the tourniquet and capillary flow may have already been past the point of peak reperfusion capillary flow. This may be supported by the fact that a visual hyperaemia was seen after both a hot bath and capsicum cream, while this was not visible after the tourniquet release.

Another limitation of our study is that the situations in which we tested hyperaemia and vasoconstriction were extreme and do not represent normal physiological situations. Probably the effect of capillary blood flow in normal day life will be much less outspoken and may be even neglectable. In previous studies presumed seasonal variations in superficial skin flow were associated with a VC of 5-6% in SAF.[11]

Implications

We conclude that a warning not to use any cream in the days before measurement and for sunscreens and sun tanners even in the 2 weeks before measurement seems necessary to assure accuracy. Furthermore, we advise to perform measurements with the arm in a normal perfusion state. The manual provided by the manufacturer of the AGE Reader already advises not to measure SAF after recent use of skin creams. Currently, attempts are made to have the device provide warnings about detection of previous possible use of skin creams, using the effects on reflectance and other spectral changes.

In earlier studies the value of SAF in prediction of diabetic complications and mortality has been well established. Also in dialysis patients, SAF has shown predictive

value on mortality. When confounding factors like body creams and extreme perfusion states of the measured arm would be cautiously eliminated, the predictive value of SAF may even surpass the earlier reported values.

Conclusion

This study aims to clarify pitfalls in the measurement of SAF. Local use of body creams can result in falsely high values of the measured SAF, accompanied by a drop in reflectance. Especially creams that absorb UVA light, chemically react with the stratum corneum or have autofluorescent properties of their own may affect SAF, persisting for days. An extremely low reflectance in a normal white skin in combination with a high SAF value should raise the suspicion of the recent use of a disturbing cream. Extreme local vasodilatation and vasoconstriction also affect SAF, but to a lesser extent, and should be avoided at the time of measurement.

Conflict of interest

A.J. Smit and R.Graaff are founder and stockholder of DiagnOptics B.V., The Netherlands, and manufacturer of the AGE-Reader, which has been used as the device for performing skin autofluorescence measurements discussed in this study.

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APPENDIX

Specifications of the contents of the creams used in this study

Body lotion

Manufactured by Etos® BV Beverwijk

Aqua, dicaprylyl carbonate, paraffinum liquidum, glycerine, stearic acid, dimethicone, cetearyl alcohol, sodium lactate, sodium PCA, glycine, fructose, urea, niacinamide, inositol, sodium benzoate, lactic acid, hydrogenated polydecene, tocopheyl acetate, parfum, phenoxyethanol, benzoic acid, dehydroacetic acid, phenethyl alcohol, PPG-2 methyl ether, methylisothiazolinone, PEG-40 hydrogenated castor oil, sodium hydroxide, tetra sodium EDTA, sodium cetearyl sulphate, acrylates/C10-30 alkyl acrylate crosspolymer, xanthan gum, maris sal.

Day cream

Manufactured by Etos® BV Beverwijk

aqua, cyclopentasiloxane, glycerin, glyceryl stearate SE, propylheptyl caprylate, cetearyl alcohol, terminalia catappa extract, propylene glycol, avena sativa kernel extract, titanium dioxide, palmitic acid, stearic acid, hibiscus abelmoschus seed extract, sambucus nigra extract, butylene glycol, tocopheryl acetate, butyrospermum parkii butter, parfum, xanthan gum, sodium acrylate/sodium acryloyldimethyl taurate copolymer, isohexadecane, retinyl palmitate, arachis hypogaea oil, PVP, polysorbate 80, acrylates/C10-30 alkyl acrylate crosspolymer, sodium hydroxide, benzyl alcohol, tannic acid, linalool, methylchloroisothiazolinone, methylisothiazolinone, CI 15985, CI17200.

Sunscreen, water resistant, spf 50

Manufactured by Etos® BV Beverwijk

Aqua, octocryleen, ethylhexyl salicylate, butyl methoxydibenzoymethane, methyleen bis-benzotriazolyl tetramethylbutylphenol, C12-15 alkyl benzoate, glycerine, glyceryl stearate, PEG 100 stearate, tricontanyl PVP, ethylhexyl triazone, parfum, cyclopentasiloxane, cetyl alcohol, bis-ethylhexyloxyphenol methoxyphenyl triazine, decyl glucoside, tocopheryl acetate, biosacheride gum-1, benzyl salicylate, xanthan gum, bisabolol, polyacrylamide, C13-14 isoparaffin, linalool, limonene, butylphenyl methylpropional, alpha-isomethyl ionone, hexyl cinnamal, disodium EDTA, laureth-7, propylene glycol, benzyl alcohol, citronellol, citric acid, PEG-8, benzyl benzoate, hydroxyisohexyl 3-cyclohexane carboxaldehyde, tocopherol, methylchloroisothiazolinone, ascorbyl palmitate, methyliso-thiazolinone, ascorbic acid.

Self browning cream

Manufactured by Vichy®

Aqua, propyleneglycol, dihydroxyacetone, ethylhexyl salicylate, ethylhexyl methoxycinnamate, C12-15 alkyl benzoate, cyclopentasiloxane, alcohol denat., glycerin, arachidyl alcohol, PEG-L00 stearate, di-C12-13 alkyl tartrate, glyceryl stearate, dimethicone, arachidyl glucoside, behenyl alcohol, butylparaben, C13-14 isoparaffin, disodium EDTA, ethylparaben, glycine soja/soybean oil, isobutylparaben, Laureth-7, methylparaben, phenoxyethanol, polyacrylamide, propylparaben, tocopherol, parfum.

Cremor capsici

Manufactured by Pharmachemie BV®

10% ethyleenglycolmonosalicylate, 0.1% histamindihydrochloride, 1% methylnicotinate, 0.1% capsicumextract (Spanish pepper extract), stearinic acid, sorbitol, triethanolamine, methylparahydrocybenzoate, cetylstearylaclcohol, sodiumlaurylsulphate.

PART III

SKIN AUTOFLUORESCENCE IN ATHEROSCLEROTIC DISEASE

Chapter 5

Skin autofluorescence is increased in patients with carotid artery stenosis and peripheral artery disease

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ABSTRACT

Objectives: Advanced glycation end products (AGEs) have a pivotal role in atherosclerosis. We evaluated skin autofluorescence (SAF), a non-invasive measurement of tissue AGE accumulation, in patients with carotid artery stenosis with and without coexisting peripheral artery occlusive disease (PAOD).

Materials and methods: SAF was measured using the AGE Reader™ in 56 patients with carotid artery stenosis and in 56 age- and sex-matched healthy controls without diabetes, renal dysfunction or known atherosclerotic disease.

Results: SAF was higher in patients with carotid artery stenosis compared to the control group: mean 2.81 versus 2.46 (p=0.002), but especially in the younger age group of 50 to 60 years old: mean 2.82 versus 1.94 (p=0.000). Patients with carotid artery stenosis and PAOD proved to have an even higher SAF than patients with carotid artery stenosis only: mean 3.28 versus 2.66 (p=0.003). Backward linear regression analysis showed that age, smoking, diabetes mellitus, renal function and the presence of PAOD were the determinants of SAF, but carotid artery stenosis was not.

Conclusion: SAF is increased in patients with carotid artery stenosis and PAOD. The univariate and multivariate associations of SAF with age, smoking, diabetes, renal insufficiency and PAOD suggest that increased SAF can be seen as an indicator of widespread atherosclerosis.

INTRODUCTION

Advanced glycation end products (AGEs) accumulate in long lived tissue during lifetime, which is regarded as a process of normal ageing. AGE accumulation results from a combination of hyperglycaemia, hyperlipaemia, oxidative/carbonyl stress and also decreased renal clearance of AGE precursors. Accelerated AGE accumulation is therefore seen in diabetes mellitus and renal failure and contributes to long term complications and mortality.[1-3] AGEs also play a major pathogenetic role in atherosclerosis.[4-7] Cross linking of AGEs with collagen and elastin within the vascular wall contributes to arterial stiffness.[1,5,6] AGEs also alter the extracellular matrix and promote atheroma formation.[6-8] Furthermore, activation of cell membrane receptors including the receptor for AGE (RAGE) leads to activation of several oxidative and inflammatory pathways.[1,7,8] This cascade leads to endothelial dysfunction, vascular inflammation and production of reactive oxygen species.[1] These mechanisms accelerate the formation of atherosclerosis.[1] Finally, AGE accumulation and overexpression of RAGE within the plaque may promote plaque instability.[9]

Accumulation of AGEs in tissue can be assessed through illumination of the skin, a technique named skin autofluorescence (SAF), which has previously been validated by simultaneous measurements of SAF and contents of specific AGE assessments in skin biopsies.[10-12] Although the fluorescent characteristics in this method are not specific for fluorescent AGE, multiple validation studies have shown convincingly and consistently that SAF has a strong correlation with specific AGE content in skin biopsies.[10-12] The correlation between SAF with the fluorescent AGE pentosidine is very high: r=0.87. Surprisingly, not only fluorescent AGE (pentosidine) but also non fluorescent AGE (N-carboxymethyl-lysine (CML) and N-carboxyethyl-lysine (CEL)) in the skin biopsies showed great correlation with SAF. Skin AGE content explained the major part of the variance (up to 76%) in the SAF signal in a pooled analysis of three validation studies.[11] We earlier reported increased SAF in several groups of patients with increased AGEs formation such as diabetes mellitus,[3,13] decreased clearance of AGEs such as renal failure[14] and overt atherosclerotic disease such as patients with stable coronary artery disease.[15] Earlier studies have already demonstrated an elevated serum level of AGEs in patients with carotid disease; a positive association between intima media thickness (IMT) and serum levels of AGEs were found in population with renal insufficiency starting dialysis.[16] Baumann et al showed that the AGE N-epsilon-carboxymethyllysine (CML) is present in the subendothelial space of atherosclerotic human carotid artery material of normoglycaemic subjects with a mean age of 50 years.[17] However, the level of SAF as a measurement of increased tissue accumulation of AGEs rather than plasma AGEs level has not yet been studied in patients with carotid artery stenosis. Therefore,

the present study evaluates SAF in patients with atherosclerotic carotid artery stenosis with or without coexisting peripheral arterial disease. The possible value of SAF as a risk indicator in this specific cohort of patients is further discussed.

MATERIALS AND METHODS

Subjects

Between October 2007 and May 2008 56 consecutive patients with carotid artery stenosis admitted to the outpatient clinic of the Department of Surgery (Division of Vascular Surgery) in our tertiary referral hospital participated in the study after informed consent was obtained. The degree of stenosis and basic morphologic features of the plaque were evaluated by duplex ultrasound. Duplex ultrasound was performed in the clinical setting as part of standard medical care. This was performed by one observer, a specialized sonographer of the department of vascular surgery of our university hospital. Inclusion criteria were the presence of a symptomatic stenosis of 70-99% (or less in case of ulcerative soft plaques) or an asymptomatic stenosis of 80-99%. There were no exclusion criteria. 56 age- and sex-matched controls were recruited at the outpatient clinic of the Department of Anaesthesiology for preoperative evaluation prior to an elective non-cardiovascular related surgical procedure. Control patients were eligible if they did not have a history of diabetes, cardiovascular disease or renal disease. Carotid ultrasound was not performed in the control group.

Study protocol

In both groups, classical cardiovascular risk factors as well as other factors that are known to influence AGE accumulation were inventoried: body mass index, smoking status, diabetes mellitus, hypertension (defined as a blood pressure of more than 140/90 mmHg or the use of antihypertensive medication), hypercholesterolemia (depicted by the use of statins), renal function and the presence of coronary artery disease and peripheral artery occlusive disease (PAOD). SAF was measured in carotid artery stenosis and control patients.

Assessment of skin autofluorescence

SAF was measured with the AGE Reader™ (DiagnOptics Technologies BV, Groningen, the Netherlands). The AGE Reader™ is a desk-top device that uses the characteristic fluorescent properties of some AGEs to estimate the level of AGE accumulation in the skin. Technical details of this non-invasive device concerning the optical technique have been described more extensively elsewhere.[11] In short, the AGE Reader illuminates a

skin surface of 4 cm^2 guarded against surrounding light with an excitation light source with a peak excitation of 370 nm. Emission light (fluorescence in the wavelength of 420-600 nm) and reflected excitation light (with a wavelength of 300-420 nm) from the skin is measured with a spectrometer. SAF is calculated as the ratio between the emission light and reflected excitation light, multiplied by 100 and expressed in arbitrary units.

In the current series of experiments, the right forearm was positioned on top of the device. The right forearm is the standard measuring site for SAF as it is the most practical site and autofluorescence is believed to be uniformly distributed throughout the body. A series of three consecutive measurements was carried out, which took less than a minute time. The mean SAF of three consecutive measurements was calculated and used in the analyses. An earlier validation study showed an intra individual Altman error percentage of 5.03% with SAF measurements taken over 1 single day and an Altman error percentage of 5.87% over seasonal variation.[11] Between subjects, SAF has a standard deviation of approximately 0.5 AU.[18] SAF shows a linear increase with age of 0.023 AU per year for subjects up to 70 years.[18] Gender had no influence on SAF in non-smokers. In smokers, SAF was 0.2 AU higher in females.[18] The SAF measurement with the AGE reader is independent of skin colour when reflectance values are above 12% as was the case in all of the study participants.[18] Measurements were performed in fasting state as previous studies showed a postprandial rise up to 12%.[19-20]

Statistical analysis

Power analysis was based on the reference values for SAF provided by Koetsier et al.[18] Using a mean SAF of 2.5 AU with a SD of 0.55 AU, 56 patients and 56 controls were needed to detect a difference of at least 0.3 AU (=12 percent) with a power of 80% and a p-value <0.05. Data were prospectively gathered in a database (SPSS 15-0, SPSS Inc, Chicago, Illinois, USA). Distribution of variables was tested by the Kolmorgorov-Smirnov test. All parameters showed a normal distribution. Descriptive statistics are therefore presented as mean with standard deviation or as number of patients. For comparison between continuous variables the t-test was used. For categorical variables the Fisher exact test was used. Correlations between variables were analysed by Pearson's correlation. Subsequently, backward linear regression analysis was performed to determine the parameters that independently influenced SAF. A P-value less than 0.05 was regarded as statistically significant.

RESULTS

Subject characteristics

Patient characteristics are summarized in table I. The mean age of both groups was approximately 69 years. All patients were Caucasian and thirty-seven patients (66%) were male in both groups. As expected, the presence of traditional cardiovascular risk factors as well as established coronary artery or peripheral artery occlusive disease (PAOD) was significantly higher in the patients with carotid artery stenosis. Twenty five patients with carotid artery stenosis were current smokers compared to 9 in the control group. Mean BMI was not different between the groups, both approximately 27.5 kg/m^2. Twelve patients (21%) had diabetes mellitus and three of them used insulin therapy. Mean blood pressure was 153/80 mmHg in the patients with carotid artery disease and 156/84 mmHg in the control group. Diastolic blood pressure was significantly higher in the control group. Nearly all patients with carotid artery stenosis had hypertension (n=54) and nearly half of them used more than two antihypertensive agents. Significantly fewer subjects in the control group had hypertension (n=43) but only 18 of these hypertensive controls received antihypertensive medication. Statins were used by 49 patients with carotid artery stenosis (87.5%), while only one subject in the control group was on statin therapy. All patients with carotid artery stenosis used either antiplatelet therapy (n=50) or an oral vitamin K inhibitor (n=6). Mean serum creatinin was 90 in the patients with carotid artery stenosis and 96 umol/liter in the control group. Nearly half of the patients with carotid artery stenosis also had either coronary artery disease or peripheral artery occlusive disease (n=26). Of the 56 patients with carotid artery disease, 14 had coexisting PAOD and 17 coexisting coronary artery disease. As a consequence of the exclusion criteria, none of the control group patients had atherosclerotic manifestations.

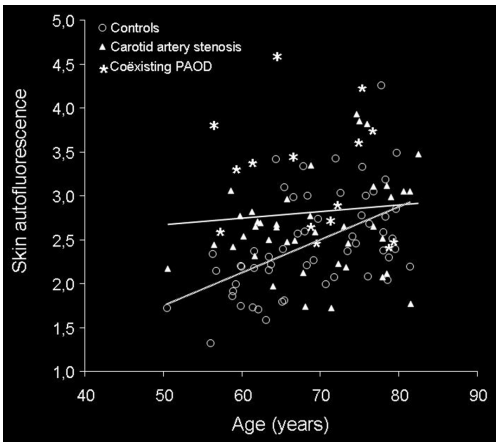
Table I: Baseline characteristics presented as mean (standard deviation), median (interquartile range) or as number of patients (%).

Characteristic	Carotid artery stenosis	Control subjects	p-value
N	56	56	
Age (years)	69.0 (7.8)	68.9 (7.9)	0.96
Male sex	37 (66%)	37 (66%)	1.0
Current smoker	25 (45%)	9 (16%)	0.002
BMI (kg/m2)	27.2 (4.9)	27.8 (3.9)	0.49
Diabetes mellitus	12 (21%)	0	0.001
Diabetes mellitus requiring insulin	3 (5%)	0	0.24
Systolic blood pressure	153 (22)	156 (22)	0.47
Diastolic blood pressure	80 (74-89)	84 (80-89)	0.016
Hypertension (>140/90 mmHg/med)	54 (96%)	43 (77%)	0.004
Antihypertensive medication	52 (93%)	18 (32%)	<0.001
>2 antihypertensive agents	22 (39%)	2 (4%)	<0.001
Statin therapy	49 (88%)	1 (2%)	<0.001
Serum creatinine	90.1 (34.5)	95.6 (15.7)	0.28
Renal clearance (MDRD)	84 (37)	73 (16.7)	0.050
Anti platelet therapy	50 (89%)	0	<0.001
Coronary artery disease	17 (30%)	0	<0.001
PAOD	14 (25%)	0	<0.001
Coronary artery disease and/or PAOD	26 (46%)	0	<0.001

Skin autofluorescence

Overall, SAF was significantly higher in the patients with carotid artery stenosis (mean SAF 2.81) compared to the control group (mean SAF 2.46) (Table II). After stratification for age, the difference in SAF was primarily observed in the age category of 50 to 60 years: a mean SAF of 2.82 in the carotid artery stenosis patients versus a mean SAF of 1.94 in the controls. (Table II). After exclusion of patients with diabetes mellitus the differences in SAF remained significant in the total group with carotid artery stenosis as well as in the age subgroup of 50 to 60 years old. Figure 1 shows that the control group exhibits the natural increase of SAF with advancing age ($r=0.52$, $p<0.001$). In the patient group with carotid artery stenosis, the relationship between SAF and age has disappeared as younger patients already have a high SAF ($r=0.094$, $p=0.49$).

Figure 1: Scatter plot showing SAF in relation to age in patients with carotid artery stenosis and controls. Circles denote control subjects, triangles patients with carotid artery stenosis, stars patients with carotid artery stenosis and coexisting PAOD.



Trend lines are shown for controls (dotted line) and carotid artery stenosis (continuous line). The control group exhibits the natural increase of SAF with advancing age. In the patient group with carotid artery stenosis, the relationship between SAF and age has disappeared. Patients with carotid artery stenosis and coexisting PAOD show a higher SAF than the control subjects and the patients with carotid artery disease only.

Within the carotid artery stenosis group, the degree of stenosis did not influence SAF (p=0.71). Also, no difference in SAF was found between the asymptomatic and symptomatic patients with carotid artery stenosis (p=0.21).

PAOD proved to be strongly associated with a high SAF. Patients with PAOD had a higher SAF than patients without peripheral arterial occlusive disease. When comparing SAF of patients with carotid artery stenosis and PAOD to SAF in the control group, SAF was 3.28 (0.66) versus 2.46 (0.57) with a p value of 0.000 (table II). Even within the group of carotid artery stenosis, SAF was significantly higher when there was coexisting PAOD. Subjects with both carotid artery stenosis and PAOD had a SAF of 3.28 (0.66) versus 2.66 (0.53) for subjects with only carotid artery stenosis without coexisting PAOD. When excluding patients with PAOD, the difference in SAF between the patients with carotid artery stenosis and controls was limited and even lost statistical significance: SAF was 2.66 (0.53) versus 2.46 (0.57) with a p value of 0.08 (table II). Between the patients with carotid artery stenosis with and without coexisting PAOD no statistical differences between baseline characteristics could be found. Unlike PAOD, the presence of coexisting coronary artery disease did not result in a higher SAF. Subjects with carotid

artery stenosis and also coronary artery disease had a SAF of 2.82 (0.69) while this was 2.81 (0.46) for patients with carotid artery disease without coexisting coronary disease.

Table II: Skin autofluorescence in patients with carotid artery stenosis compared with healthy controls.

Skin autofluorescence	Carotid artery stenosis	N=	Control subjects	N=	p-value
Total group	2.81 (0.62)	56	2.46 (0.57)	56	0.002*
Total group without diabetes	2.75 (0.64)	44	2.46 (0.57)	56	0.016*
Total group with PAOD	3.28 (0.66)	14	2.46 (0.57)	56	0.000*
Total group without PAOD	2.66 (0.53)	42	2.46 (0.57)	56	0.084
Total group with CAD	2.82 (0.46)	17	2.46 (0.57)	56	0.021*
Total group without CAD	2.81 (0.69)	39	2.46 (0.57)	56	0.008*
50-60 years	2.82 (0.54)	8	1.94 (0.30)	10	0.000*
50-60 years without diabetes	2.79 (0.37)	5	1.94 (0.30)	10	0.000*
60-70 years	2.73 (0.58)	23	2.40 (0.54)	21	0.06
70-80 years	2.90 (0.71)	21	2.73 (0.55)	24	0.37
>80 years	2.83 (0.74)	4	2.19 (n=1)	1	0.49

Analysis of different age groups. Peripheral artery occlusive disease and coronary artery disease are denoted by respectively PAOD and CAD**.** Statistical significance is notated by *.

Correlations and univariate analysis

Univariate analysis and correlations were performed in the combined total group to determine the relationship of different parameters with SAF. A significant correlation of SAF was found with age ($r=0.29$, $p<0.01$) and with renal function ($r=0.24$, $p=0.01$). A significantly higher SAF was found in current smokers (mean 3.01 versus mean 2.47, $p<0.001$), diabetes mellitus (mean 3.03 versus 2.59, $p=0.02$), those using statins (mean 2.87 versus 2.44, $p<0.001$), those with hypertension (mean 2.73 versus 2.48, $p=0.04$) and those with coexistence of PAOD (mean 3.28 versus 2.46, $p<0.001$). Gender, BMI, hypertension and coronary artery disease had no significant influence in univariate analysis.

Backward linear regression analysis

Backward linear regression analysis was performed in the combined total group to determine the parameters that independently contributed to SAF. The results are shown in table III. Age, smoking, diabetes mellitus, renal function and presence of PAOD were

the significant determinants of SAF. In contrast to the results of univariate analysis the presence of carotid artery disease did not contribute significantly with a β of 0.074 (p=0.83). Coronary artery disease also did not contribute with a β of 0.038 (p=0.48).

Table III: Results of backward linear regression analysis.

Included parameter	βeta	p-value
Age	0.24	0.02
Current smoker	0.36	0.00
Diabetes mellitus	0.21	0.01
EGFR (MDRD)	0.20	0.05
Peripheral artery occlusive disease	0.29	0.00

The remaining significant predictors of skin autofluorescence are shown. Carotid artery disease, coronary artery disease, sex, BMI, hypertension and use of statin medication did not significantly influenced skin autofluorescence. Carotid artery disease and coronary artery disease both did not contribute significantly with respectively a β of 0.074 (p=0.83) and 0.038 (p=0.48).

DISCUSSION

In the current study we showed that SAF is significantly elevated in patients with carotid artery stenosis and PAOD compared to controls. Two findings were especially remarkable.

First, PAOD proved to be an important determinant of SAF. High SAF values were especially found in the group with carotid artery disease and coexisting PAOD. Even within the group of carotid artery disease, SAF was significantly higher when there was coexisting PAOD with a SAF of 3.36 versus 2.64 (p=0.003). This could not be explained by differences in baseline characteristics between the patients with carotid artery stenosis with or without PAOD. Also in linear regression analysis, PAOD proved to be a strong determinant of SAF, even more so then diabetes. This finding is interesting and warrants further research. It suggests that SAF should primarily be seen as an indicator of widespread atherosclerotic disease. Currently, a study analysing SAF in patients with primary peripheral artery disease has been initiated.

The second remarkable finding was that SAF was specifically elevated for patients in the age group 50 to 60 years. Furthermore, the relation between age and SAF, as normally seen, was present in the control group but absent in the patients with carotid artery stenosis.

The marked elevation of SAF levels in carotid artery stenosis was demonstrated in non-diabetic patients as well as in diabetics. A high SAF in diabetes mellitus may therefore be regarded as an indicator of widespread atherosclerotic disease and not only a manifestation of diabetes mellitus per se. Mulder et al. previously reported similar findings in a cohort with stable coronary artery disease.[15] SAF was significantly increased in stable coronary artery disease compared with controls, irrespective of diabetes, current smoking and renal function. Earlier studies in patients with diabetes mellitus also showed that SAF is manifestly increased in these patients. The level of AGE accumulation correlated with the duration and the grade of complications of diabetes.[2,3,21]

Univariate and multivariate analysis of associations with SAF in the present study supports this interpretation in our study group of patients with carotid artery stenosis. SAF was univariately associated with age, smoking, diabetes mellitus, renal dysfunction, dyslipidaemia and the presence of carotid artery disease and peripheral arterial occlusive disease which is in concordance with earlier studies.[2,3,11,14] Multivariately, SAF was determined by age, smoking, diabetes mellitus, renal dysfunction and peripheral arterial occlusive disease. Therefore, again, increased SAF may rather be regarded as an indicator of widespread atherosclerotic disease than as a specific identifier of carotid atherosclerotic disease.

This study however has several limitations. At baseline, despite matching for sex and age in the control group, there were differences between the patient and control groups including presence of diabetes, smoking behaviour, hypertension, use of antihypertensive medication and use of statins. This may be considered a source of confounding. However, it does represent the expected increased presence of risk factors for cardiovascular disease in patients with carotid artery stenosis compared to healthy people.

In the control group asymptomatic atherosclerosis may have existed as no carotid ultrasound or other vascular tests were performed in the control group. If asymptomatic atherosclerosis was present in the control group this would result in an underestimation of the difference between the control group and patient group. The differences we found between the control group and the group with atherosclerotic carotid stenosis and peripheral artery disease therefore would be even greater in a better selected control group.

Our study is also limited by the small number of patients. The fact that backward linear regression did not show carotid artery disease or coronary artery disease to be independent determinants of SAF may therefore be caused by the lack of power. Mulder et al has earlier shown that SAF indeed is elevated in stable coronary disease.[15] A large study evaluating the relationship between SAF en intima media thickness is

currently being executed to further clarify this issue. What may be the use of the present results? The correlation of SAF with traditional cardiovascular risk factors, the presence of diabetes, renal insufficiency and peripheral arterial occlusive disease could indicate that SAF may be an indicator of high cardiovascular risk patients. Moreover, since SAF represents end organ damage it may predict morbidity and mortality more than the classical risk factors for atherosclerosis separately. For patients with type 2 diabetes this has already been established since SAF added prognostic information to the UKPDS risk calculator in predicting mortality.[22] Strong predictive results of SAF for cardiovascular mortality were also seen in patients with renal failure.[23] The same may be true for patients with carotid artery stenosis and peripheral artery disease. Prospective follow-up studies are necessary to elucidate this. Moreover, spectroscopy techniques show promising results for imaging vulnerable plaques and the near future will tell whether they really shine light on unstable cardiovascular disease.

In conclusion, skin autofluorescence is increased in patients with carotid artery stenosis and PAOD compared to healthy controls. SAF is especially elevated in the age group of 50 to 60 years, suggesting an increased accumulation of tissue AGEs in these patients. The univariate and multivariate associations of SAF with age, smoking, diabetes mellitus, presence of renal insufficiency and peripheral arterial occlusive disease suggest that increased SAF should primarily be seen as an indicator of widespread atherosclerotic disease. These associations further underscore the important role of AGEs in the pathophysiology of atherosclerotic disease. SAF might therefore be an indicator of an overall high burden of widespread atherosclerosis as a consequence of AGE accumulation. Future research should investigate the use of SAF as a superior predictor of cardiovascular events in this population.

Conflict of interest

A.J. Smit is founder of DiagnOptics B.V., Groningen, The Netherlands, manufacturer of the AGE Reader™, which has been used to perform skin autofluorescence measurements as reported in this manuscript.

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Chapter 6

Skin autofluorescence as a measure of advanced glycation endproducts deposition is elevated in peripheral artery disease

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ABSTRACT

Objective: Evidence for an important role of Advanced Glycation Endproducts (AGEs) in the development of atherosclerosis and cardiovascular disease beyond diabetes mellitus (DM) and renal disease is growing. Skin autofluorescence (SAF) is a validated non-invasive measure of tissue AGEs. We hypothesized that SAF is elevated in peripheral artery disease (PAD).

Methods and Results: A case control study was performed in 492 patients with PAD and 164 controls, matched for age (mean 66±10 years) and presence of DM. Cardiovascular risk factors and co-morbidity (coronary artery disease, cerebrovascular disease, abdominal aortic aneurysm (AAA)) were assessed. SAF was measured with the AGE Reader™. SAF was higher in patients compared to controls: geometric mean 2.77 (95% CI: 2.71-2.83) versus 2.44 (95% CI: 2.35-2.53) AU, $p=0.4 \times 10^{-8}$. In logistic regression, the adjusted odds-ratio for presence of PAD was 2.47 (95% CI: 1.66-3.69) per 1 unit increase of SAF. PAD patients with cardiovascular co-morbidity had a higher SAF as compared to those without: geometric mean 2.93 (95% CI: 2.85-3.02) versus 2.63 (95% CI: 2.55-2.71) AU, $p=0.4 \times 10^{-6}$, also after correction for confounders. Regression analysis showed that age, smoking, DM, chronic kidney disease and a history of cerebrovascular disease or AAA were independently associated with SAF in the PAD patients.

Conclusion: Accumulation of tissue AGEs is increased in PAD patients, independent of cardiovascular risk factors and co-morbidity, although these conditions are associated with a further increase. These findings underscore the importance of AGEs in PAD, irrespective of the presence of diabetes and renal insufficiency.

INTRODUCTON

Patients with peripheral artery disease (PAD) are inflicted with a large burden of atherosclerosis, associated with a substantially increased risk for cardiovascular events and premature mortality.[1-3] Advanced glycation endproducts (AGEs) are formed by non-enzymatic glycation and oxidative reactions to form stable structures accumulating on long-lived proteins and promote cellular stress responses by engagement of the receptor for AGEs (RAGE).[1] Although formerly only implicated in diabetes mellitus and renal disease, evidence for an important role of AGEs in cardiovascular disease beyond these conditions is growing. AGEs have been immunohistochemically localized in human atherosclerotic lesions and lowering AGEs or blocking AGE-receptors (RAGE) in murine models attenuates plaque formation, supporting a causal role of AGEs in the development of atherosclerosis.[4-6] Indeed, in patients with peripheral artery disease (PAD), elevated levels of plasma AGEs have been documented.[7]

Skin autofluorescence (SAF) is a validated measure of tissue AGEs that can be used in subjects with a skin pigmentation up to Fitzpatrick type V.[8-10] Earlier, we reported that SAF was increased and a strong predictor of mortality in diabetes mellitus and end stage renal disease.[9,11,12] The aim of the present study was to compare SAF in PAD patients with controls. We hypothesized that SAF is increased in PAD. Furthermore, we explored the relation of SAF with the traditional cardiovascular risk factors as well as with additional cardiovascular damage expressed by a history of cerebrovascular and/or coronary artery disease and/or an abdominal aortic aneurysm in these patients.

METHODS

Study population

We performed a case-control study. Men and women at least 18 years of age were eligible to participate. PAD was ascertained by a resting ankle-brachial index (ABI) ≤0.90 or a toe-brachial index ≤0.70 in case of noncompressible calf arteries. PAD was confirmed by evidence of obstructive disease on computed tomographic angiography, magnetic resonance angiography, catheter angiography and/or duplex ultrasonography. An age-matched control group was selected from patients without a history of PAD that visited the outpatient clinic for pre-operative evaluation for orthopedic or minor surgical procedures. Since diabetes mellitus and end-stage renal disease strongly increase AGEs levels, PAD patients with diabetes mellitus were matched with diabetic controls and patients with end-stage renal disease (chronic kidney disease (CKD)[13] stage 5; estimated GFR<15 ml/min/1.73 m^2) were excluded from both groups. Additional

exclusion criteria for patients and controls were recent myocardial infarction, stroke or sepsis (all within the past 3 months), cancer and solid organ transplantation. Patients and control subjects were not matched by gender, since no difference in SAF was found between men and women in previous studies.[8,9,11,12] We designed the study to have 3:1 matching (patients:controls) in order to have sufficient patients for additional analyses (see statistical analysis). For each set of three patients with approximately the same age (maximum deviation of 1 year from their mean age) one age-matched control subject was selected.

The study was approved by the local institutional review board and all participating subjects gave informed consent.

Evaluation

For data collection, history was taken and medical records were reviewed. Traditional cardiovascular risk factors were assessed: age, gender, smoking status, body mass index (BMI), diabetes mellitus, hypertension and hypercholesterolemia. BMI was calculated as weight (kilogram) divided by squared height (meters). Patients were classified as having hypertension based on use of blood pressure lowering drugs and hypercholesterolemia was assigned in case of current use of lipids lowering drugs. The American Diabetes Association criteria were used to diagnose diabetes mellitus.[14] Serum creatinine was determined and renal function was estimated by calculating the glomerular filtration rate (eGFR).[15] Renal insufficiency was classified according to CKD stage.[13] Anticoagulant therapy was defined as the use of antiplatelet or vitamin K antagonist therapy. Coronary artery disease (CAD) was evaluated by clinical history of myocardial infarction, angina pectoris, coronary artery surgery and/or percutaneous coronary intervention. Cerebrovascular disease (CVD) was ascertained by history of symptoms of transient ischemic attack and/or stroke. An aneurysm of the abdominal aorta (AAA) was defined as a previously documented maximum infrarenal aortic diameter of ≥30 mm. For PAD patients without CAD, 10 year risk of a coronary event was estimated according to the Framingham risk score. This score is depending on age, total cholesterol, HDL-cholesterol, systolic and diastolic blood pressure, presence of diabetes and smoking.[16] Patients were classified into three risk categories depending on gender and Framingham risk score: low (<10%), intermediate (10 to 20%), or high (>20%) 10 year risk of having a coronary event. Since this score is validated for subjects <75 year, all patients ≥75 year were excluded for this calculation.

Skin autofluorescence

SAF was measured with the AGE Reader™ (DiagnOptics Technologies BV, Groningen, the Netherlands). The AGE Reader is a desk-top device that uses the characteristic

fluorescent properties of certain AGEs to estimate the level of AGEs accumulation in the skin. Technical details of this non-invasive device concerning the optical technique have been described more extensively elsewhere.[8] In short, the AGE Reader illuminates a skin surface of 4 cm^2 guarded against surrounding light with an excitation light source with a peak excitation of 370 nm (ultraviolet A). Emission light (fluorescence in the wavelength of 420-600 nm) and reflected excitation light (with a wavelength of 300-420 nm) from the skin is measured with a spectrometer. SAF is calculated as the ratio between the emission light and reflected excitation light, multiplied by 100 and expressed in arbitrary units (AU). In previous validation studies using skin biopsies, we showed a strong correlation between SAF and the skin contents of the fluorescent AGE pentosidine as well as with the non-fluorescent AGEs Nε-(carboxymethyl)-lysine (CML) and Nε-(carboxyethyl)lysine (CEL).[8,9] Also, a strong correlation between SAF measurements of the arm and leg was found in a validation study. In the current study, the right forearm was positioned on top of the device which is the standard and most practical measuring site for SAF. A series of three consecutive measurements was carried out, taking less than a minute. The mean SAF was calculated from these three measurements and used in the analyses. An earlier validation study showed an intra individual Altman error percentage of 5.03% with SAF measurements taken over 1 single day and an Altman error percentage of 5.87% for seasonal variation.[8]

Statistical analysis

All data are shown as number (percentage) for categorical variables, as mean (± standard deviation) for variables with a normal distribution and as geometric mean (95% confidence interval) in case of a non-normally distributed parameter that was normalized by logarithmic transformation. Normal distribution was tested by a one sample Kolmogorov-Smirnov test. Characteristics of patients and control subjects were compared by use of the χ^2 test for categorical variables and Student independent t-test for continuous variables. SAF between patients and controls was compared by Student independent t-test. Within the group of PAD patients, the effect of the presence of each traditional cardiovascular risk factor, the presence of additional cardiovascular damage (expressed by a history CAD, CVD or AAA), and the effect of ABI and Framingham risk score category on SAF was tested in a univariate analysis by Student independent t-test or one-way ANOVA where applicable. A p-value of <0.05 was considered statistically significant. Backward linear regression was used to identify the independent determinants of SAF within the group of PAD patients. In the complete study group (patients and control subjects), logistic regression was performed to study the association between SAF and the presence of PAD. All statistical analyses were carried out with the Statistical Package for Social Science (SPSS, version 18.0).

RESULTS

Characteristics of patients and control subjects

Five-hundred and ten patients with PAD were willing to participate. Eighteen patients were excluded, 7 patients because of renal failure and 11 patients because of a kidney transplantation. The remaining 492 PAD patients and 164 control subjects, with a mean age of 66±10 years, were included. In 91 patients, PAD was not ascertained by a resting ABI ≤0.90 because of noncompressible arteries (33 patients) or because invasive angiography and/or surgery had directly been performed (58 patients). In all of these cases, PAD was confirmed by angiography. The characteristics of patients and controls are shown in table I. These characteristics are shown for the complete group of PAD patients, as well as for the two subgroups of PAD patients with and without a history of cardiovascular co-morbidity (CAD, CVA and/or AAA). As expected, the traditional cardiovascular risk factors were more prevalent in the PAD patients than in the control subjects. Male gender, current smoking, hypertension and hypercholesterolemia were more common in the PAD patients ($p \leq 0.001$ for all). PAD patients had a slightly, but statistically significant higher eGFR (77 (95% CI: 74-79) versus 72 (95% CI: 69-75) ml/min/1.73 m^2, $p=0.02$) and a lower BMI ($p<0.001$). Diastolic blood pressure was lower in PAD patients ($p<0.01$), probably due to the more frequent use of blood pressure lowering drugs. Obviously, PAD patients used oral anticoagulants more often ($p<0.001$). CAD and AAA were more prevalent in PAD patients (both $p<0.001$), but the prevalence of CVD did not differ between PAD patients and control subjects.

Skin autofluorescence

SAF was higher in PAD patients as compared to the control subjects: geometric mean 2.77 (95% CI: 2.71-2.83) versus 2.44 (95% CI: 2.35-2.53) AU, ($p=0.4x10^{-8}$), see figure 1. The univariate influence of the presence of traditional risk factors, cardiovascular co-morbidity and of the ABI on SAF is shown in figure 2. Age, presence of diabetes mellitus, renal function and a history of CAD, CVD or AAA had a significant effect on SAF. Gender, current smoking, presence of hypertension and of hypercholesterolemia, BMI and ABI were not correlated with SAF. The results of the multivariate linear regression analysis with SAF as dependent variable are shown in table II. Age, current smoking, diabetes mellitus, CKD class and a history of CVD or AAA were independently associated with SAF. The variables gender, hypertension, BMI, hypercholesterolemia, a history of CAD and ABI did not independently contribute to SAF.

Table I: Baseline characteristics.

Characteristics		Controls	Peripheral artery disease (PAD) patients	P-value controls versus PAD patients
N	Total group	164	492	
No CV co-morbidity			*252*	
With CV co-morbidity			*240*	
Age in years	Total group	65.8 (10.4)	65.9 (10.5)	N/A
No CV co-morbidity			*63.0 (11,1)*¶	
With CV co-morbidity			*68.8 (8,9)*	
Male gender	Total group	88 (54%)	345 (70%)	0.0001
No CV co-morbidity			*155 (62%)*¶	
With CV co-morbidity			*190 (79%)*	
Current smoker	Total group	37 (23%)	251 (51%)	0.2×10^{-9}
No CV co-morbidity			*150 (60%)*¶	
With CV co-morbidity			*101 (42%)*	
Body Mass Index in kg/m^2 *	Total group	27.8 (27.1-28.5)	26.3 (25.9-26.7)	0.0002
No CV co-morbidity			*25.8 (25.2-26.4)* #	
With CV co-morbidity			*26.9 (26.4-27.4)*	
Diabetes mellitus	Total group	42 (26%)	127 (26%)	N/A
No CV co-morbidity			*54 (21%)***	
With CV co-morbidity			*73 (30%)*	
Systolic BP (mmHg) †	Total group	149 (23)	146 (25)	NS
No CV co-morbidity			*147 (25)*	
With CV co-morbidity			*145 (25)*	
Diastolic BP (mmHg) ‡	Total group	82 (11)	79 (14)	0.002
No CV co-morbidity			*81 (15)***	
With CV co-morbidity			78 (14)	
Hypertension	Total group	67 (41%)	403 (82%)	0.5×10^{-23}
No CV co-morbidity			*181 (72%)*¶	
With CV co-morbidity			*222 (93%)*	
Hypercholesterolemia	Total group	48 (29%)	372 (76%)	0.9×10^{-26}
No CV co-morbidity			*173 (69%)*¶	
With CV co-morbidity			*199 (83%)*	
Oral anticoagulant therapy	Total group	53 (32%)	436 (89%)	0.1×10^{-45}
No CV co-morbidity			*209 (83%)*¶	
With CV co-morbidity			*227 (95%)*	

Characteristics		Controls	Peripheral artery disease (PAD) patients	P-value controls versus PAD patients
eGFR (ml/min/1.73m^2) §	Total group	72 (69-75)	77 (74-79)	0.02
No CV co-morbidity			*83 (80-87)*¶	
With CV co-morbidity			*71 (67-74)*	
Ankle-brachial index ||	Total group	N/A	0.57 (0.14)	N/A
No CV co-morbidity			*0.57 (0.14)*	
With CV co-morbidity			*0.57 (0.15)*	
Coronary artery disease (CAD)	Total group	23 (14%)	168 (34%)	0.9 x10^{-6}
No CV co-morbidity			*0 (0%)*¶	
With CV co-morbidity			*168 (70%)*	
Cerebrovascular disease (CVD)	Total group	27 (17%)	76 (15%)	NS
No CV co-morbidity			*0 (0%)*¶	
With CV co-morbidity			*76 (32%)*	
Abdominal arterial aneurysm (AAA)	Total group	0 (0%)	61 (12%)	0.2 x10^{-5}
No CV co-morbidity			*0 (0%)*¶	
With CV co-morbidity			*61 (25%)*	

Data presented as numbers of patients (%), mean (SD) or as geometric mean (95% CI). Cardiovascular (CV) co-morbidity defined as a history of CAD and/or CVA and/or AAA. eGFR=glomerular filtration rate by MDRD formula. Patients and controls were matched on age and presence of diabetes mellitus. BP=blood pressure. NS=non-significant. N/A=not applicable.

* Data missing for 1 control patients and 5 PAD patients, BMI was natural log transformed for analysis.

† Data missing for 1 control patients and 2 PAD patients.

‡ Data missing for 1 control patients and 4 PAD patients.

§ Data missing for 13 control patients and 6 PAD patients, eGFR was natural log transformed for analysis.

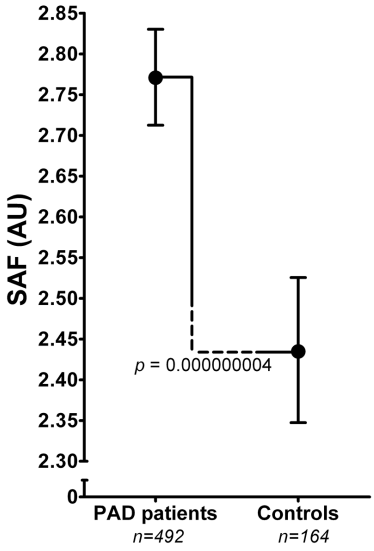
|| Data missing for 91 PAD patients.

PAD patients with versus without CV co-morbidity, ¶ p<0.001, # p<0.01, ** p-value<0.05.

Table I also shows the characteristics of PAD patients in two subgroups: patients with and without additional cardiovascular co-morbidity (history of CVD and/or CAD and/or AAA). Patients with a history of cardiovascular co-morbidity were older, more often male, less frequently smokers, had a higher BMI, more often diabetes mellitus, hypertension and/or hypercholesterolemia, used oral anticoagulant therapy more often, had a lower eGFR and a lower diastolic blood pressure as compared to PAD patients without cardiovascular co-morbidity. Systolic blood pressure and ABI did not differ between these groups. Figure 3 (left panel) shows the influence of an increasing

burden of atherosclerosis on SAF, with a history of CVD and/or CAD and/or AAA as marker of additional atherosclerotic burden. There was a significant difference in SAF between patients with PAD without cardiovascular co-morbidity and patients with PAD and cardiovascular co-morbidity: 2.63 (95% CI: 2.55-2.71) versus 2.93 (95% CI: 2.85-3.02) AU, p=0.4 x10^{-6}. The difference in SAF between control subjects and PAD patients without cardiovascular co-morbidity was significant as well as with PAD patients with cardiovascular co-morbidity: 2.44 (95% CI: 2.35-2.53) versus 2.63 (95% CI: 2.55-2.71) AU, p=0.002 and 2.44 (95% CI: 2.35-2.53) versus 2.93 (95% CI: 2.85-3.02) AU, p=0.7 x 10^{-13} respectively. After correction for gender and age, SAF remained significantly different between PAD patients with and without cardiovascular co-morbidity (p=0.0005) as well as between controls and PAD patients without cardiovascular co-morbidity (p=0.0001) and between controls and PAD patients with cardiovascular co-morbidity (p=0.1 x10^{-11}). Figure 3 also shows the relation between Framingham risk score category and SAF (right panel) in PAD patients without CAD and <75 year of age. Patients with a high risk had a higher SAF as compared to patients with an intermediate or low risk: 3.03 (2.86-3.20) vs 2.62 (2.51-2.73) and vs 2,54 (2.38-2.70), p=0.00006 and p=0.00005, respectively.

Figure 1: Skin autofluorescence (SAF) in peripheral artery disease patients and control subjects. Data are shown as geometric mean (95% CI).



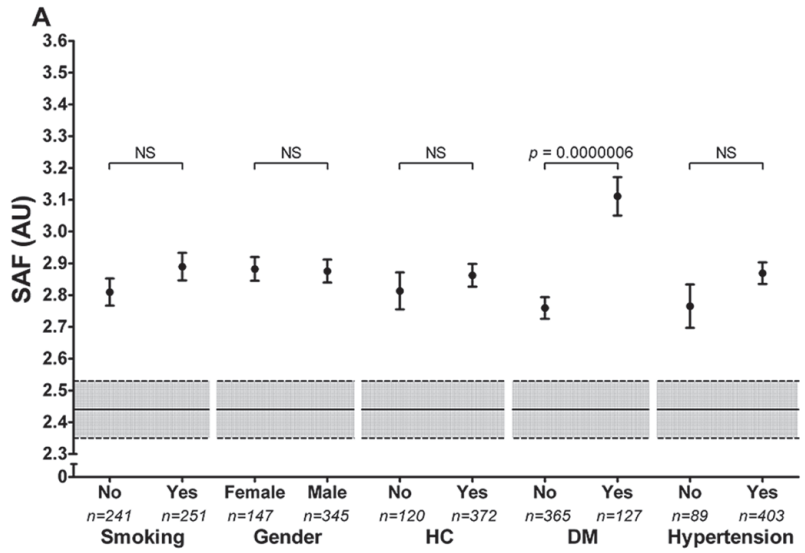
AU=arbitrary units.

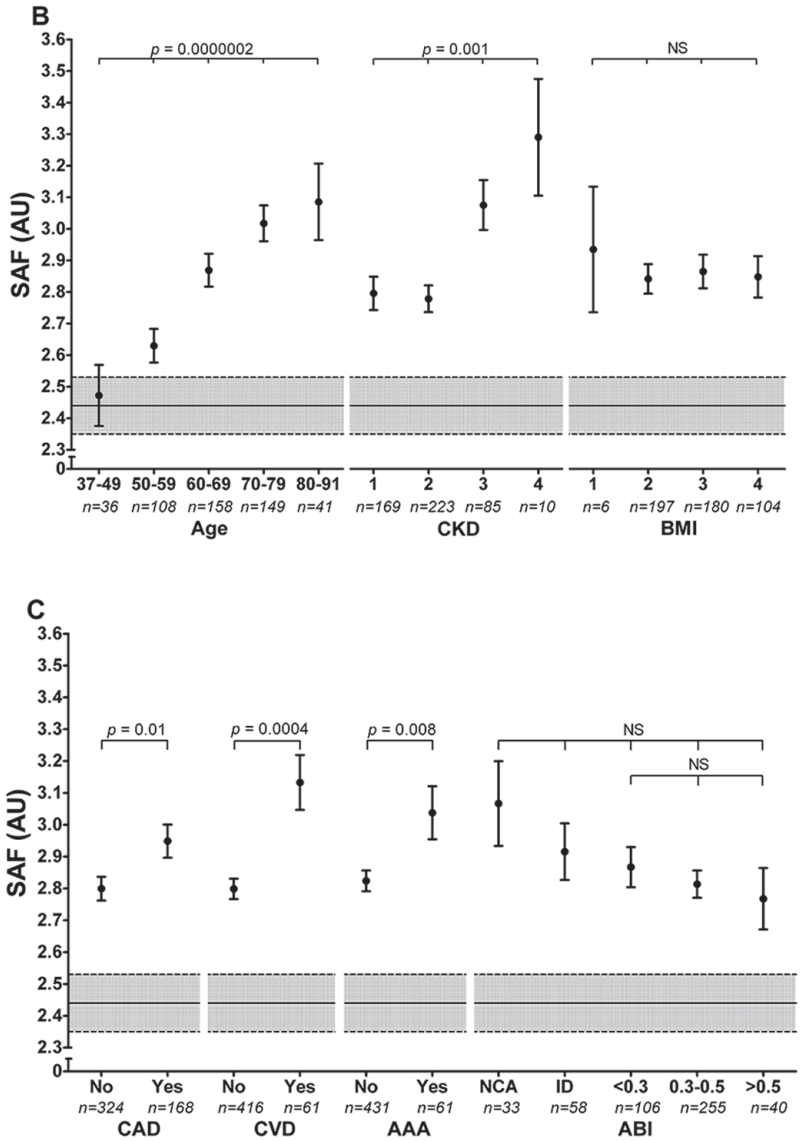
Table II: Multivariate model of (natural log of) skin autofluorescence in the peripheral artery disease patients: result of backward linear regression analysis.

Variable	Coefficient B	Standard error	Standardized coefficient β	P-value
Age	0.005	0.001	0.227	0.000004
Smoking	0.097	0.022	0.195	0.00002
Diabetes Mellitus	0.113	0.024	0.204	0.000003
Natural log eGFR	-0.061	0.030	-0.092	0.044
Cerebrovascular disease	0.073	0.029	0.109	0.011
Abdominal aortic aneurysm	0.070	0.032	0.095	0.028

Variables removed from the model: gender, natural log BMI, hypertension, hypercholesterolemia, coronary artery disease, ankle-brachial index.
eGRF = estimated glomerular filtration.

Figure 2: Association of skin autofluorescence (SAF) with cardiovascular risk factors (dichotomous variables are shown in panel A: smoking, gender, hypercholesterolemia, diabetes mellitus and hypertension; categorical variables are shown in panel B: age, CKD class and BMI class) and cardiovascular morbidity and ankle-brachial index (panel C: CAD, CVD, AAA and ankle-brachial index) in patients with peripheral artery disease (PAD). Data are shown as geometric mean (95% CI).





AU=arbitrary units. The gray area represents the geometric mean (95% CI) SAF of the control group. NS=not significant. HC=hypercholesterolemia. DM=diabetes mellitus. Age category by decade, except for the lowest category consisting of all patients <50 years and the highest category consisting of all patients ≥80 years. CKD: chronic kidney disease classification (see text). Patients without kidney disease are combined with patients in CKD 1. CKD data are missing for 6 PAD patients. BMI=Body mass index; class 1=underweight, BMI <18.5 kg/m^2; class 2=normal weight, 18.5≤ BMI <25.0 kg/m^2; class 3=overweight, 25.0 ≤ BMI <30 kg/m^2; class 4=obesity, BMI ≥30 kg/m^2. BMI data are missing for 5 PAD patients. CAD=coronary artery disease. CVD=cerebrovascular disease. AAA=abdominal aortic aneurysm. ABI=ankle-brachial index; NCA=group with noncompressible arteries, ID=group with directly invasively diagnosed PAD without ABI measuement.

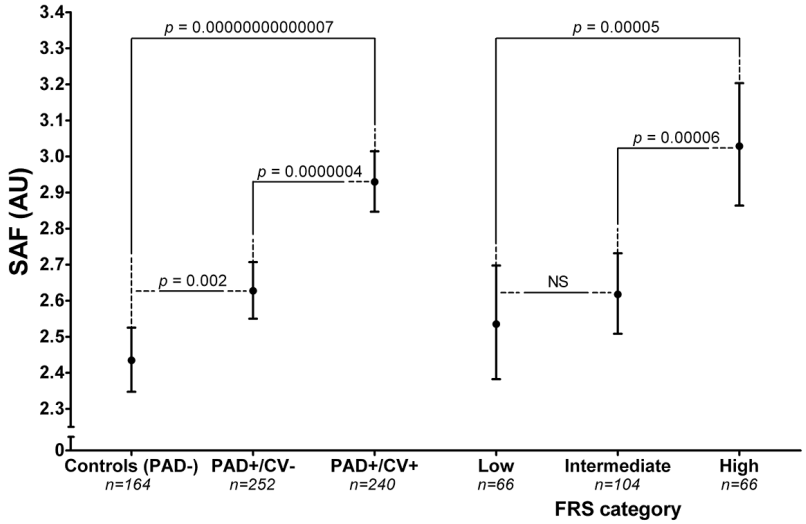
Table III: Logistic regression models for the association between peripheral artery disease (PAD) and skin autofluorescence (SAF).

Variable	B	Standard error	Odds Ratio (95% CI)
Crude model			
SAF	0.85	0.16	2.34 (1.73-3.17)
Constant	-1.17	0.41	0.31
Model 1			
SAF	0.86	0.20	2.36 (1.59-3.50)
Male gender	0.71	0.24	2.03 (1.26-3.26)
Smoking	1.45	0.28	4.25 (2.48-7.23)
Hypertension	1.56	0.27	4.78 (2.82-8.09)
Hypercholesterolemia	1.64	0.25	5.13 (3.12-8.43)
BMI	-0.09	0.03	0.91 (0.87-0.96)
eGFR	0.01	0.01	1.01 (1.00-1.02)
Constant	-2.95	1.09	0.05
Model 2			
SAF	0.82	0.16	2.27 (1.66-3.09)
CVD	-0.40	0.26	0.67 (0.40-1.12)
CAD	1.08	0.25	2.95 (1.81-4.80)
Constant	-1.27	0.42	0.28
Model 3			
SAF	0.91	0.20	2.47 (1.66-3.69)
Male gender	0.73	0.25	2.07 (1.26-3.37)
Smoking	1.47	0.28	4.35 (2.53-7.47)
Hypertension	1.58	0.28	4.85 (2.80-8.39)
Hypercholesterolemia	1.66	0.27	5.28 (3.12-8.92)
BMI	-0.09	0.03	0.91 (0.86-0.95)
eGFR	0.01	0.01	1.01 (1.00-1.02)
CVD	-1.07	0.33	0.34 (0.18-0.65)
CAD	0.39	0.32	1.48 (1.80-2.75)
Constant	-2.85	1.10	0.06

eGFR=estimated glomerular filtration rate by MDRD formula. BMI=body mass index. CVD=cerebrovascular disease, CAD=coronary artery disease. Since PAD patients and controls were matched for age and presence of diabetes mellitus, these variables were not included in the model. For gender, female is the reference. AAA was excluded from the model; no estimation of the odds ratio is possible since there are no control subjects with an AAA.

Table III shows the results of the logistic regression models that describe the association between SAF and the presence of PAD in the complete group (patients and controls). In the crude model, each 1 AU increase of SAF was associated with a 2.34 fold (95% CI: 1.73-3.17) increased chance of having PAD. The strength of this association was unaffected by correction for cardiovascular risk factors (model 1, OR 2.36 (95% CI: 1.56-3.50)), history of CAD and CVD (model 2, OR 2.27 (95% CI: 1.66-3.09)) and cardiovascular risk factors and history CAD and CVD (model 3, OR 2.47 (95% CI: 1.66-3.69)).

Figure 3: Data are shown as geometric mean and 95% confidence interval (CI).



AU=arbitrary units. NS=not significant. Left panel: skin autofluorescence (SAF) in peripheral artery disease (PAD) patients with and without cardiovascular co-morbidity (cardiovascular and/or cerebrovascular disease and/or abdominal aortic aneurysm) and control subjects (PAD-). PAD/+CV–=PAD patients without cardiovascular co-morbidity. PAD+/CV+=PAD patients with cardiovascular co-morbidity. Right panel: SAF in PAD patients without coronary artery disease (n=324) with a low (<10%), intermediate (10 to 20%), or high (>20%) 10 year risk of a coronary event according to the Framingham risk score (FRS). FRS could be calculated for 236 patients. For 88 patients, FRS could not be calculated because of a high age (>74 years) or missing blood pressure or cholesterol values.

DISCUSSION

Our study in patients with PAD shows that SAF, as a measure of tissue AGEs accumulation, is considerably increased compared to control subjects, independent of cardiovascular risk factors and cardiovascular co-morbidity. Still, the presence of cardiovascular risk factors known to be strongly associated with PAD further increases SAF in PAD patients. Multivariate analysis shows that age, current smoking, presence of diabetes mellitus, eGFR and a history of CVD or AAA are independent determinants of SAF in patients with PAD. This is in agreement with our previous studies. Furthermore, a 1 unit increase in SAF is associated with a 2.45 fold higher chance of having PAD, independent of cardiovascular risk factors and cardiovascular co-morbidity in the complete group.

Advanced glycation end products in PAD

This is the first report demonstrating an increased SAF in PAD patients, although we earlier reported that increased SAF in patients with carotid artery disease was confined to those with PAD.[17] The role of AGEs in the pathogenesis of PAD has recently been suggested, which is in line with the more established concept of AGEs as important players in the development of atherosclerosis in diabetes mellitus, renal insufficiency and coronary artery disease.[9,11,12,18]

The literature on AGEs in PAD, however, is relatively scarce. Previously, in a study of patients with end stage renal disease, plasma levels of S100A12, which is a ligand for the receptor for AGEs (RAGE), where elevated in the patients with PAD as compared to those without PAD.[19] Furthermore, a direct inverse association between ABI and circulating levels of the AGE pentosidine was found in apparently healthy men.[20] However, no patients with documented PAD or an ABI below 0.9 were included. In contrast, we included PAD patients with a mean ABI of 0.57 and did not find any relation between SAF and ABI in the univariate analysis. Also the multivariate model showed that in our PAD patients, SAF was primarily depending on age, smoking, DM and renal function rather than the severity of PAD as assessed by the ABI. Interestingly, we did find that SAF was particularly increased in those PAD patients that had additional cardiovascular morbidity, univariately as well as in the multivariate model. Therefore, in these patients, AGEs may reflect the burden of atherosclerosis with incremental AGEs accumulation in those patients in whom atherosclerosis is not confined to the peripheral arteries but extends to the aorta, coronary or cerebral arteries as well. In line with this concept of increasing AGEs with increased manifestation of atherosclerosis are the results of a postmortem study in patients with diabetes mellitus demonstrating a stronger expression of RAGE in patients with an increased necrotic plaque area in the coronary arteries.[21]

In our study, logistic regression analysis showed that each 1 AU increase of SAF was associated with a 2.34 fold increased chance of having PAD in the crude model. The independency of SAF as a determinant of the presence of PAD was underscored by the unchanged strength of their association in models that include an increasing number of known determinants of the presence of PAD such as smoking, hypertension and the presence of CAD. The main outcome of our study that shows an increased SAF in PAD as well as the additional analyses further reinforce the concept of a key role for AGEs in development of atherosclerotic disease, in particular PAD.

Limitations of the study

The selection of PAD patients and controls may have influenced the results. The controls were not selected on being healthy, but only on being free of symptoms or signs of PAD. However, if there have been control patients with PAD at a subclinical level, this has resulted in an underestimation rather than an overestimation of the true difference in SAF between patients with PAD and control subjects. Furthermore, patients and controls were matched for age and the presence of diabetes mellitus, because in earlier studies these were the most important determinants of SAF.[8,9,11,12,17,18] This, again, may have lead to an underestimation of the true difference in SAF between PAD and controls. The same is true for the exclusion of patients with end stage renal disease (CKD class 5, on dialysis, of after kidney transplantation). We had to exclude 18 patients and none of the control subjects because of this criterion, while end stage renal disease is known to impair clearance of AGEs resulting in accelerated accumulation of AGEs.

Generalisability of the results may be limited to a part of all PAD patients. The low mean ABI of 0.57 demonstrates that our group of PAD patients suffered from severe PAD, especially when taking into account that the ABI was not measured in 58 patients because invasive angiography and/or surgery had directly been performed, necessitated by the clinical presentation of these patients with critical ischemia. The high prevalence of most cardiovascular risk factors in the PAD patients of our study may be expected in a cohort with established vascular disease. The high percentages of patients with hypertension or hypercholesterolemia in the patients with PAD can be explained by the fact that these conditions are known risk factors for PAD, but also by the definition we used for these conditions i.e. the use of antihypertensive or cholesterol lowering drugs. These drugs may have been prescribed as secondary cardiovascular prevention measures rather than because of the presence of hypertension or hypercholesterolemia.

Also, we did not perform skin biopsies in the present study to confirm the strong correlation between SAF and skin content of AGEs, as reported in earlier validation studies in different patient groups and healthy controls.[8,9] Furthermore, we did not measure plasma AGEs to corroborate our findings. However, it is unclear whether

sampling of plasma AGES would have been useful, since blood and urine sampling of AGEs is hampered by the fact that these AGEs not necessarily reflect tissue AGE levels.[22,23]

A major limitation of the use of skin autofluorescence by the AGE reader to measure AGEs level is that it cannot be used in all types of skin. For a valid measurement of skin autofluorescence the reflectance of excitation light has to be at least 6%.[10] Strongly pigmented skin type absorbs too much excitation light, resulting in a reflectance of less than 6%. In practice, the AGE reader can be used subjects with a skin pigmentation up to Fitzpatrick skin type V. Therefore, the AGE reader can be used in subjects with a Caucasian, Mediterranean, Hispanic, Asian or Eastern Indian or origin, but not in African Americans.

Finally, we do not know the strength of the correlation between SAF and actual level of AGEs in the atheroma of affected arteries. A study on the agreement between SAF as measure of tissue AGEs in the skin and tissue AGEs or plaque size in arteries has yet to be performed. Still, in patients with type 1 diabetes mellitus, another research group has established the positive association between coronary artery calcification score and skin intrinsic fluorescence (SIF) measurements that in essence use the same fluorescent properties of AGEs in the skin as the AGE reader we used.[24] Also, a recently published study showed a correlation between SAF and the actual content of AGEs in residual bypass graft material.[25] SAF as a non-invasive method to quantify tissue accumulation of AGEs has made it possible to study 656 subjects in the present study relatively easily, not needing skin biopsies or atheroma of affected arteries.

Clinical implications

Notwithstanding these limitations, our observation of highly increased SAF in PAD patients remains relevant, while the use of SAF as a measure of tissue AGEs deposition has been validated earlier and used as such in a series of prospective clinical studies as well.[8] In several of these studies SAF was shown to be a strong predictor for cardiovascular morbidity and mortality and total mortality, independent of conventional cardiovascular risk factors.[9,11,12,17,18] If such a predictive value can be established in PAD patients, SAF may be used to identify PAD patients at highest risk for cardiovascular events. Our observation of a relation between SAF and Framingham risk score category supports the possibility of the use of SAF for risk prediction in PAD patients. However, this has yet to be shown in a prospective follow-up study. Likewise, whether PAD patients with a high SAF may benefit from more aggressive treatment (i.e. tighter control of cardiovascular risk factors with lower LDL-cholesterol and blood pressure targets), similar to patients with diabetes mellitus or renal insufficiency, should be subject to an intervention study. Finally, inhibition of AGE formation, breakdown of AGE-crosslinks and blocking the receptor for AGEs may become treatment targets in cardiovascular

disease. Drugs that are currently prescribed to treat diabetes mellitus, hypertension and hypercholesterolemia may have such properties to a certain extent, but also new drugs have specifically been designed to counter AGEs.[26] However, although some of these new drugs have already been tested in clinical studies, efficacy and safety concerns have prevented these drugs from their use in clinical practice.

Conclusions

Skin autofluorescence, as a measure of tissue advanced glycation endproducts deposition, is considerably increased in patients with PAD as compared to controls. This increase is independent of the presence of cardiovascular risk factors or other established cardiovascular disease although these conditions are associated with a further increase of SAF. These findings underscore the importance of AGEs in PAD, irrespective of the presence of diabetes and renal insufficiency.

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Disclosures

Smit A.J. is founder of DiagnOptics BV, the Netherlands, manufacturing autofluorescence readers (http://www.diagnoptics.com/). The other authors have nothing to declare.

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Chapter 7

Skin autofluorescence and risk of micro- and macrovascular complications in patients with Type 2 diabetes mellitus – a multicentre study

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ABSTRACT

Aims: Skin autofluorescence is a non-invasive marker of advanced glycation end product accumulation. In a previous study, skin autofluorescence correlated with and predicted micro- and macrovascular complications in Type 2 diabetes in a primary care setting. The present cross-sectional study aims to confirm the association between skin autofluorescence and diabetic complications in patients with Type 2 diabetes in a multi-centre secondary care setting.

Methods: We analysed 563 subjects with Type 2 diabetes mellitus from five Dutch hospitals.

Results: Median age was 64 years, median duration of diabetes 13 years and median HbA_{1c} 58 mmol/mol (7.5%). Sixty-one per cent of patients had microvascular complications (38% nephropathy, 36% retinopathy, 35% neuropathy) and 42% had macrovascular complications. Median UK Prospective Diabetes Study 10-year risk for coronary events was 19%. Median skin autofluorescence was elevated compared with age-matched healthy control subjects: 2.77 (interquartile range 2.39-3.28) vs. 2.46 (2.08–2.84) arbitrary units. Skin autofluorescence was particularly increased in patients with complications: no complications, median 2.56 (2.26-2.90); microvascular complications, 2.79 (2.38-3.29); macrovascular complications, 2.85 (2.41-3.41); both micro- and macrovascular complications, 2.96 (2.56-3.60) arbitrary units, $P<0.001$. Logistic regression analysis showed that age, duration of diabetes, renal function, gender, atrial fibrillation and skin autofluorescence were independently associated with macrovascular complications. Multiple regression analysis identified age, smoking, renal function, macrovascular complications and the number of microvascular complications as the determinants of skin autofluorescence.

Conclusions: This study confirms that skin autofluorescence is increased in patients with Type 2 diabetes in a secondary care setting. Skin autofluorescence was associated with macrovascular complications in patients with diabetes and this association was independent of classical risk factors.

INTRODUCTION

Advanced glycation end products accumulate in tissues with a low turnover during a lifetime.[1,2] This is regarded as a process of normal ageing. The accumulation of accelerated advanced glycation end products results from combinations of hyperglycaemia, hyperlipaemia, oxidative/carbonyl stress and also decreased renal clearance of advanced glycation end product precursors.[1,2] Highly accelerated advanced glycation end product formation and accumulation is seen in diabetes mellitus.[1] This is considered one of the important pathogenetic mechanisms causing end organ damage in diabetes.[1] Cross-linking of proteins by advanced glycation end products and receptor-mediated cellular activation contribute to loss of elasticity of the vascular wall and to cellular inflammation, resulting in micro- and macrovascular complications.[1,3] The contents of the advanced glycation end products in skin biopsies predicted long-term diabetic complications in a large cohort of patients with Type 1 diabetes, even after adjustment for HbA1c.[4,5]

Skin autofluorescence has a strong correlation with the specific advanced glycation end product content in skin biopsies, as shown by multiple validation studies.[6-8] Not only fluorescent advanced glycation end products (pentosidine), but also non-fluorescent advanced glycation end products such as *N*-carboxymethyl-lysine and *N*-carboxyethyl-lysine in the skin biopsies correlated with skin autofluorescence.[6] We therefore consider skin autofluorescence a non-invasive marker of tissue accumulation of advanced glycation end products. Earlier studies showed that skin autofluorescence is increased in Type 2 diabetes compared with healthy control subjects.[9] Skin autofluorescence showed a strong association with the severity of diabetes-related complications.[9,10] Furthermore, it also predicted both micro- and macrovascular complications in patients with Type 2 diabetes.[10] In a previous prospective follow-up study, skin autofluorescence had an additional value to the UK Prospective Diabetes Study (UKPDS) risk score for estimating mortality in Type 2 diabetes mellitus.[11] However, these findings concern one single-centre cohort of well-controlled patients with Type 2 diabetes in a primary care setting. Our study aims to confirm the relation between skin autofluorescence and diabetic complications and possibly broaden our insight into the role of skin autofluorescence in predicting micro- and macrovascular complications. We evaluated skin autofluorescence in a group of patients with Type 2 diabetes in a secondary medical care setting. Furthermore, we chose a multi-centre approach to possibly support the generalizibility wider use of the results. Here, we present the baseline cross-sectional data. Prospective follow-up data on the predictive value of skin autofluorescence on macrovascular complication will be presented in the future.

SUBJECTS AND METHODS

Subjects

We recruited 616 subjects with Type 2 diabetes mellitus from five hospitals in different regions of the Netherlands. The University Medical Centre Groningen (UMCG), Medical Centre Leeuwarden, Onze Lieve Vrouwe Gasthuis Amsterdam, Sint Franciscus Gasthuis Rotterdam and Het Diakonessenhuis Meppel participated in this study. Patients were included from April 2007 to November 2009. Informed consent was obtained from all participants. The study was approved by the Medical Ethical Committee.

Assessment of skin autofluorescence

Skin autofluorescence was measured with the advanced glycation end product (AGE) Reader™ (DiagnOptics Technologies BV, Groningen, the Netherlands). The AGE Reader is a desktop device that uses the characteristic fluorescent properties of some advanced glycation end products to estimate the level of advanced glycation end product accumulation in the skin. Technical and optical details of this non-invasive method have been described more extensively elsewhere.[8] In short, the AGE Reader illuminates a skin surface of 4 cm^2, guarded against surrounding light, with an excitation light source with a peak excitation of 370 nm. This wavelength is in the UVA spectrum. Emission light in the wavelength range of 420-600 nm (fluorescence) and excitation light that is reflected by the skin with a wavelength range of 300-420 nm from the skin is measured with a spectrometer. Skin autofluorescence was determined from the ratio between the emission light and the reflected excitation light, using the AGE Reader software, version 2.2. Each participating medical centre was equipped with an AGE Reader and given user instructions for skin autofluorescence measurement. Measurements were performed by a diabetes nurse or research assistant. Skin autofluorescence was measured at room temperature while patients were at rest in a seated position. In the current series of experiments, the forearm was positioned on top of the device in the usual manner, as described by the manufacturer. Measurements were not specifically performed in a fasting state. For each skin autofluorescence value, three consecutive measurements were carried out at three different skin sites of the same forearm, within a total test period of approximately 2 min. The mean of these three consecutive measurements was used in the analyses. Skin pigmentation influences the measurement of skin autofluorescence and its influence has been extensively studied and reported earlier.[12] For a reliable skin autofluorescence measurement, skin reflectance had to be above 12% with the hardware and software (version 2.1) of the AGE Reader that was used. Patients with a dark-coloured skin with a reflectance below 12% were excluded from the analysis.

Study protocol

Clinical data from the participating subjects were gathered from the electronic medical data system of the different hospitals. Data on age, gender, diabetes duration, blood pressure and macrovascular events were collected from the medical records. HbA_{1c}, a fasting lipid profile, serum creatinine and urinary microalbumin were received from the laboratories. The standard creatinine assays of the individual hospitals laboratories were used and they were all well standardized with validated assays. Glomerular filtration rate (GFR) was estimated by the abbreviated Modification of Diet in Renal Disease (MDRD) equation: $186 \times (\text{creatinine}/88.4)^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$. The standard stages of chronic kidney disease (1–5) were used to classify renal insufficiency. Diabetic nephropathy was scored as an albumin:creatinine ratio >3.5 in women and >2.5 in men on two successive samples, or at least once in the previous year while using an angiotensin-converting enzyme (ACE) inhibitor. Diabetic retinopathy was scored according to the report of the yearly evaluation by the patient's ophthalmologist with retinal photography or fundoscopy. Diabetic neuropathy was scored by testing with a 5.07 Semmes-Weinstein monofilament applied 10 times on three areas of each foot in a random order. An absent sensation of the monofilament of three or more times (i.e. a score of lower than 8 of 10) was considered neuropathy. Microvascular disease was defined by either the presence of microalbuminuria and/or retinopathy (at least background retinopathy) and/or neuropathy. The presence of macrovascular disease was defined as a known history of coronary disease, cerebrovascular disease or peripheral vascular disease. Cardiovascular disease was defined as a history of ischaemic heart disease [International Classification of Diseases (ICD) codes I20-25] and/or a history of percutaneous coronary intervention or coronary bypass surgery. Cerebrovascular disease was defined as a history of an ischaemic cerebrovascular accident (ICD codes 63-64). Peripheral vascular disease was defined as a history of claudication with abnormal ankle-arm index (<0.8 on a single measurement or <0.9 on repeated measurements) or a history of surgical intervention of peripheral artery disease. Future cardiovascular risk of the individual patient was calculated by the UKPDS risk score. The following variables are used for risk calculation: age, duration of diabetes, gender, atrial fibrillation, ethnicity, smoking behaviour, HbA_{1c}, systolic blood pressure, total cholesterol and HDL cholesterol These provided a measure of the 10-year risk of fatal and non-fatal coronary and cerebrovascular events for each patient. As the UKDPS score incorporates these 10 cardiovascular risk factors, we used the UKPDS score as a measure of cardiovascular risk.

Statistical analysis

We calculated sample size with a power of 80%, α=0.05. We adopted the standard deviation of 0.5 from the literature.[13] We wanted to be able to demonstrate a difference

in skin autofluorescence of 0.2 arbitrary units between groups with no diabetic complications, microvascular complications, macrovascular complications and combined micro- and macrovascular complications. Power analysis showed that 77 subjects were needed in each group. Data were gathered in a database (SPSS 15-0; SPSS Inc., Chicago, IL, USA). Normal distribution of variables was assessed by Kolmorgorov-Smirnov tests. Descriptive statistics are presented as mean with standard deviation in the case of normal distribution, otherwise as median with interquartile range or as number of patients. A paired Student *t*-test was used for normally distributed variables and a paired Mann-Whitney *U*-test was used for variables with a skewed distribution. Multiple linear regression analysis was used to determine independent relations between skin autofluorescence and clinical data, including age, duration of diabetes, gender, atrial fibrillation, ethnicity, smoking behaviour, HbA_{1c}, systolic blood pressure, cholesterol and the presence of micro- and macrovascular complications. Logistic regression analysis was used to determine which variables determined presence of macro- and microvascular disease. Analysis of variance (ANOVA) was applied to compare differences between groups. For cross tabulation, χ^2 analysis was used.

RESULTS

Subject characteristics

A total of 616 patients were measured. Skin autofluorescence measurements proved to be unreliable with the software version used in this study in 53 patients, mainly because of low reflectance caused by dark pigmented skin. Therefore, 563 patients with Type 2 diabetes were included in our analysis. Baseline characteristics are presented in Table 1. The median diabetes duration was 13 years and glycaemic control was suboptimal. Blood pressure and cholesterol were reasonably well controlled. A number of 126 patients (24%) had renal insufficiency with a chronic kidney disease stage 3 or more. As expected, the number of subjects with diabetic complications was high in this secondary medical care setting: 61% had microvascular complications and 42% macrovascular complications.

Skin autofluorescence

Skin autofluorescence values for the total group and different subgroups are given in Table 2. Median skin autofluorescence was 2.77 with an interquartile range of 2.39-3.28 for the total group. This is elevated and, specifically, in the 79th percentile compared with reference values in healthy control subjects, where for this median age a median skin autofluorescence of 2.46 (interquartile range 2.08-2.84) would be

Table I: Baseline characteristics presented as mean (standard deviation), median (interquartile range) or as number of patients (%).

Characteristic	T2DM
Age (years)	64 (11.3)
Male sex	269 (47.9%)
Caucasian	517 (91.3%)
Atrial fibrillation	41 (7.3%)
Current smoker	97 (17%)
Diabetes duration (years)	13.0 (7-19)
HbA_{1c} (mmol/mol, %)	58 (51-67), 7.5%
Systolic blood pressure (mmHg)	140 (130-150)
Total cholesterol (mmol/l)	4.2 (3.6-4.9)
HDL cholesterol (mmol/l)	1.2 (1.0-1.4)
GFR (ml/min)	75 (61-92)
CKD	
none	83 (16%)
stage 1	57 (11%)
stage 2	253 (49%)
stage 3	117 (22.5%)
stage 4	9 (2%)
stage 5	0
Diabetic nephropathy	195 (38%)
Diabetic retinopathy	189 (36%)
Diabetic neuropathy	161 (35%)
Microvascular disease	346 (61%)
Coronary artery disease	169 (30%)
Cerebrovascular disease	74 (13%)
Peripheral artery disease	85 (15%)
Total macrovascular disease	238 (42%)
Risk coronary events UKPD	19% (12-30)
Risk cerebrovascular events UKDPS	13% (6-29%)

expected.[13] Table 2 shows that skin autofluorescence slowly rises with increasing age, which is regarded as a physiological phenomenon of ageing.[13] Skin autofluorescence levels increased significantly with increasing diabetic complications ($P<0.001$). In subjects without any diabetic complications, median skin autofluorescence was 2.56 (interquartile range 2.26-2.90), which is the 65th percentile for healthy

control subjects of the same age. With microvascular complications, median skin autofluorescence was 2.79 (interquartile range 2.38-3.29 and 80th percentile). In a univariate analysis, no differences in skin autofluorescence values were found between patients with one, two or three microvascular complications (ANOVA, *P*=0.12). With macrovascular complications, median skin autofluorescence was 2.85 (interquartile range 2.41-3.41 and 82th percentile) and, in those with both micro- and macrovascular complications, median skin autofluorescence was 2.96 (interquartile range 2.56-3.60, 88th percentile). The differences in skin autofluorescence between patients without diabetic complications, with microvascular complications and with both micro- and macrovascular complications remained significant after correction for age (*P*=0.008).

Table II: Skin autofluorescence according to microvascular and macrovascular complications and in different age groups (median with interquartile range).

	Skin autofluorescence	N
Total group of T2DM	2.77 (2.39-3.28)	563
T2DM without any complications	2.56 (2.26-2.90)	141
T2DM with microvascular complications	2.79 (2.38-3.29)	181
T2DM with macrovascular complications	2.85 (2.41-3.41)	71
Both micro- and macrovascular complications	2.96 (2.56-3.60)	169
<50 years	2.37 (2.00-2.76)	55
50-60 years	2.63 (2.30-3.03)	120
60-70 years	2.77 (2.36-3.20)	195
70-80 years	3.08 (2.61-3.50)	142
>80 years	3.09 (2.57-3.87)	51

Association between skin autofluorescence, micro- and macrovascular complications

Multiple linear regression analysis was performed to evaluate which variables were associated with skin autofluorescence. Up to 19% in the variance of skin autofluorescence could be explained by age (β=0.28, *P*<0.000), smoking (β=0.18, *P*<0.000), renal function (eGFR) (β=-0.13, *P*<0.006), macrovascular complications (β=0.11, *P*=0.009) and number of microvascular complications (β=0.081,*P*=0.045). Medical centre, gender, duration of diabetes, HbA1c, systolic blood pressure and lipid profile did not significantly influence skin autofluorescence. When we entered nephropathy, neuropathy and retinopathy separately in the regression analysis, none of them were significant.

Subsequently, we analysed which variables were associated with the presence of macrovascular disease. Logistic regression analysis showed age (odds ratio 1.03, *P*=0.05), renal function (eGFR) (odds ratio 0.98, *P*=0.008), diabetes duration (odds ratio 1.03, *P*=0.01), gender (odds ratio 0.51, *P*=0.001), atrial fibrillation (odds ratio 0.39, *P*=0.02) and skin autofluorescence (odds ratio 1.45, *P*=0.023) to be independently associated with the presence of macrovascular disease. Medical centre, HbA_{1c}, lipid profile, smoking, systolic blood pressure and microvascular disease, however, were not independently associated with the presence of macrovascular disease.

Predictors of the presence of microvascular disease were duration of diabetes (odds ratio 1.05, *P*=0.002), renal function (eGFR) (odds ratio 0.98, *P*<0.000) and skin autofluorescence (odds ratio 1.60, *P*=0.004). HbA_{1c}, age, gender, smoking, systolic blood pressure and lipid profile did not contribute significantly.

Table III: Percentage of macrovascular complications according to different tertiles SAF and tertiles UKPDS risk score.

% Macrovascular complications		Tertiles SAF			
		1rst	2nd	3rd	total
Tertiles	1rst	20.2	27.9	37.8	***26.2***
UKPDS	2nd	35.6	34.9	50.7	***40.7***
risk score	3rd	51.3	56.9	66.3	***59.9***
	total	***31.6***	***40.2***	***55.1***	

Macrovascular diabetic complications by UKPDS risk score and skin autofluorescence

Regression analysis showed that skin autofluorescence was independently associated with the presence of macrovascular complications. Subsequently, we divided the patients into tertiles of UKPDS risk score and tertiles of skin autofluorescence. In these cross-sectional data, we used the UKPDS risk score as a measure of cardiovascular risk, integrating classical cardiovascular risk factors and glycaemic control. For the different tertiles, we analysed the prevalence of macrovascular complications. Results are shown in Table 3. Within each tertile of skin autofluorescence, the prevalence of macrovascular complications increased significantly. Macrovascular complications rose from 32% in the 1st skin autofluorescence tertile to 55% in the 3rd tertile (*P*<0.000). For the tertiles according to UKPDS risk score, the same phenomenon was seen: with each tertile, macrovascular complications were significantly higher (*P*<0.000). Interestingly, within

each tertile of UKPDS risk score, skin autofluorescence appeared to further determine the presence of macrovascular complications. When focusing on the 1st UKPDS tertile, macrovascular complications were present in 20% in the 1st skin autofluorescence tertile, while this was 38% in the 3rd skin autofluorescence tertile (*P*=0.04).

DISCUSSION

Skin autofluorescence was previously shown to predict micro- and macrovascular complications in one prospective study in a single-centre cohort of well-controlled patients with Type 2 diabetes in the primary care setting.[9,10] In this cohort, skin autofluorescence even proved to add predictive information on cardiovascular prognosis to the UKPDS risk score.[11]

Our present multi-centre study in the secondary medical setting confirms that skin autofluorescence is indeed increased in patients with Type 2 diabetes mellitus. Furthermore, it confirms that skin autofluorescence levels have a graded relation with the presence of micro- and macrovascular complications. Our cohort had a relatively long duration of diabetes of 13 years and a reasonable, although not optimal, glycaemic control. The prevalence of micro- and macrovascular diabetic complications, as well as UKPDS risk score, were somewhat higher, but not much different than previously described in studies concerning skin autofluorescence in Type 2 diabetes mellitus.[9-11] The cohort we describe is surprisingly similar to the cohort in the primary care setting that Lutgers and co-workers previously presented.[9-11] Age, sex, smoking behaviour, creatinine clearance, micro- and macrovascular disease and skin autofluorescence values were similar in both populations. Our cohort had a longer diabetes duration (13 vs. 4 years), a marginally higher HbA_{1c} [58 vs. 53 mmol/mol (7.5 vs. 7.0%)], a better controlled systolic blood pressure (140 vs. 146 mmHg) and better controlled total cholesterol (4.2 vs. 5.2 mmol/l).

In our study, we show that skin autofluorescence was independently associated with the presence of both micro- and macrovascular complications. Surprisingly, most classical cardiovascular risk factors and glycaemic control were not significantly associated with the presence of macrovascular disease. Macrovascular disease was predicted by age, diabetes duration, renal function (eGFR), gender, atrial fibrillation and skin autofluorescence. Specifically, HbA_{1c}, blood pressure and lipid profile did not contribute. Earlier studies showed that HbA_{1c} and skin autofluorescence have a limited relation,[14] suggesting that skin autofluorescence is only partly determined by the components of the slow Maillard reaction.

When, in our present cohort, the UKPDS risk score and skin autofluorescence were both divided into tertiles, there was an increasing percentage of patients with macrovascular complications within each tertile of UKPDS risk score (as an integration of the classical risk factors and glycaemic control), going from the 1st to the 3rd skin autofluorescence tertile. The association between prevalence of macrovascular complications, UKPDS risk score and skin autofluorescence, which we present now on cross-sectional data, has no value in prediction of future cardiovascular events. The results, however, do show that the prevalence of macrovascular disease is not only associated with a high classical cardiovascular risk profile (estimated by the UKPDS risk score), but, in addition, is associated with an increased skin autofluorescence. The prospective follow-up data of our study on cardiovascular events are required to confirm that skin autofluorescence has additive value in predicting future cardiovascular disease events on top of the UKPDS risk score. These data will be presented in the future.

Our results also contribute to establish the generalizability of skin autofluorescence results in patients with Type 2 diabetes concerning the association between skin autofluorescence with diabetic complications. First of all, within our study, no differences in skin autofluorescence results were found between the five participating medical centres. Also, the levels of skin autofluorescence in these subgroups, according to diabetic complications, are very similar to the skin autofluorescence levels found in the population with Type 2 diabetes previously described by Lutgers *et al*.[9] Their study group found a skin autofluorescence of 2.77 in a population of nearly the same age as our group. Without any complications, mean skin autofluorescence was 2.57, with microvascular complications mean skin autofluorescence was 2.71 and with both micro- and macrovascular complications mean skin autofluorescence was 3.12. This is exactly the same grading of skin autofluorescence according to diabetic complications that we found in the present study. Chabroux *et al*[15] also found very high skin autofluorescence values in a cohort of patients with Type 1 diabetes with a median age of 30 years and a diabetes duration of 17 years. Without diabetic complications, subjects had a skin autofluorescence of 1.86 (which is the 93th percentile adjusted to age) and, for subjects with both retinopathy and nephropathy, skin autofluorescence was 2.94 (which is the 99.9th percentile). This study in young patients with Type 1 diabetes cannot directly be compared with results found in patients with Type 2 diabetes, but is does again show that skin autofluorescence is elevated in patients with diabetes and even significantly more when microvascular complications exist.

Limitations

Our current results are based on cross-sectional data, which are by definition inferior to prospective data. This study has, however, been designed as a prospective follow-up study. Follow-up data on complications and mortality will be presented in the future.

Macrovascular disease was defined as a known history of coronary disease, cerebrovascular disease or peripheral artery disease. We did not screen all patients for macrovascular disease by, for example, coronary angiogram or computed tomography cerebrum of ankle-arm index. Therefore, asymptomatic macrovascular disease may have been missed in our study.

The used version of the AGE Reader in this study was limited by an influence of skin pigmentation. Skin reflectance had to be above 12% for an accurate reading. This led to inaccurate skin autofluorescence readings in 53 patients who therefore could not be included in the analysis. Development of AGE Reader is still ongoing and adjustments in the device have in the meantime improved the accuracy of readings in patients with high skin pigmentation.[12]

Conclusions

This study confirms in a multi-centre secondary care setting that skin autofluorescence is increased in patients with Type 2 diabetes and has a graded relation with the presence of micro- and macrovascular complications. In Type 2 diabetes, skin autofluorescence appears to be independently associated with the presence macro- and microvascular complications, in addition to the well-known classical risk factors of the UKPDS risk score.

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Competing interests

AJS is founder and stockholder of DiagnOptics Technologies BV, the Netherlands, manufacturer of the AGE Reader, which has been used as the device for performing skin autofluorescence measurements discussed in this study. The other authors have nothing to declare.

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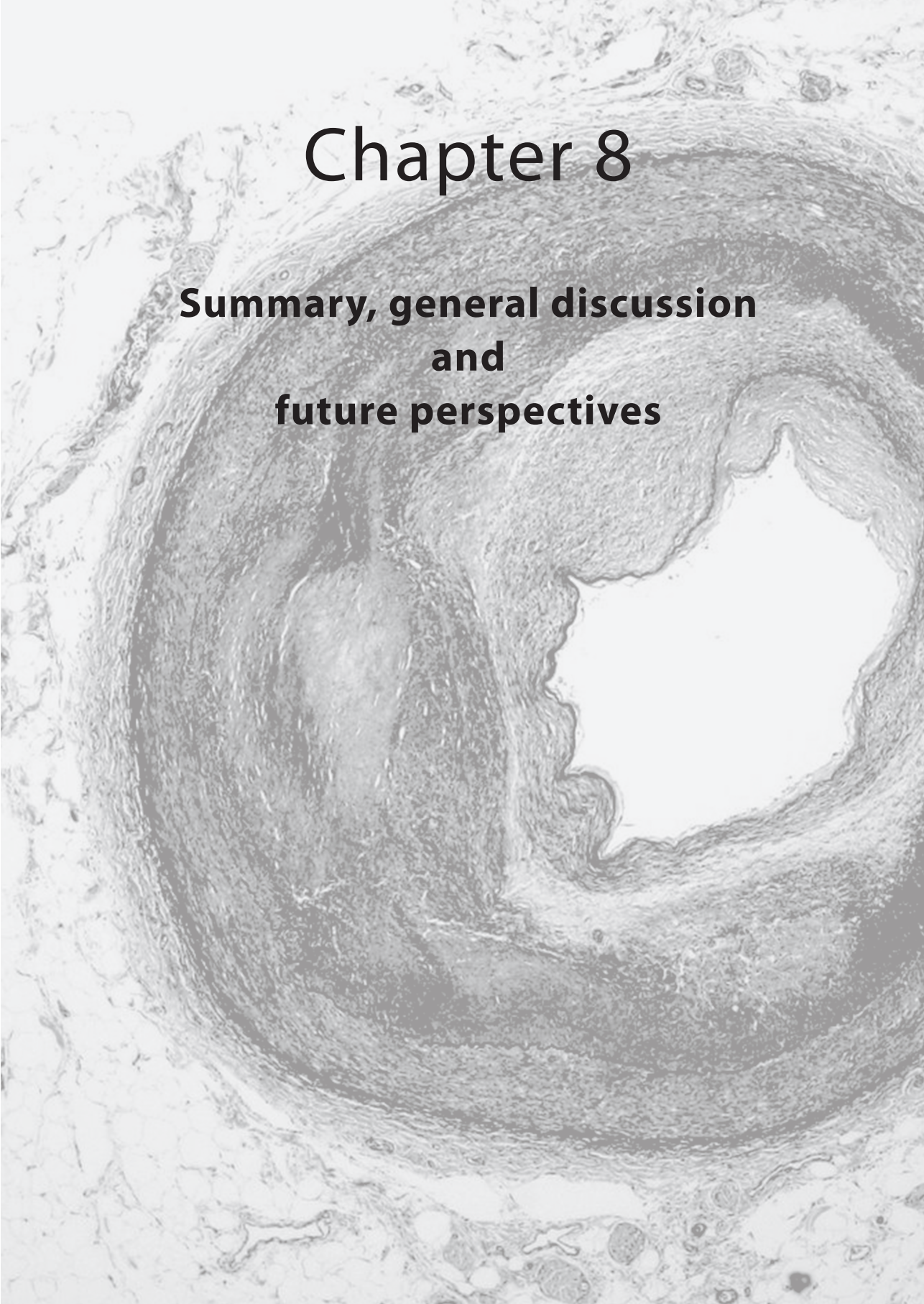

Chapter 8

Summary, general discussion and future perspectives

SUMMARY

Part 1 gives an introduction to advanced glycation end products and skin autofluorescence with chapter 2 focussing on their role in renal failure. Advanced glycation end products are formed during hyperglycemia, oxidative stress and inflammation. A complex and diverse process causes glycation and oxidation of mostly proteins, but also lipids and DNA in the body. The end product is called advanced glycation end product (AGE). This process causes irreversible structural changes and deformation of proteins, lipids and DNA leading to malfunction. Furthermore, AGE can activate pro-inflammatory receptors, especially the receptor for AGE (RAGE) with negative consequences. In tissues with a long lifetime, AGE continue to accumulate during life.

An increased accumulation of AGE is seen in diabetes mellitus (as a result of hyperglycemia), renal failure (as a result of oxidative stress and decreased renal clearance of AGE precursors) and chronic inflammatory diseases (as a result of activation of inflammatory cells).

The increased accumulation of AGE seen in these conditions plays a major pathogenetic role and leads to long-term cardiovascular complications and mortality. AGE contribute to an accelerated progression of atherosclerosis by several mechanisms. Crosslinking of vascular collagen leads to arterial stiffness, hypertension and vessel leakage. Dysfunction of AGE modified lipids also causes a negative cascade. AGE interact with their receptors on endothelial cells, smooth muscle cells and macrophages inducing adhesion molecules and cytokine expression. AGE can reduce the release of nitric oxide or captivate nitric oxide. In patients with type 2 diabetes, skin autofluorescence (SAF) predicts cardiac mortality and the occurrence of micro- and macrovascular complications. In patients on haemodialysis, SAF is highly elevated and predicts mortality. After renal transplantation SAF is lower than during haemodialysis, but still remains elevated and is a strong risk factor for chronic renal transplant dysfunction and mortality.

Measuring AGE and AGE accumulation is notoriously difficult, also because it is a heterogeneous group. Since 2006 AGE accumulation can be non-invasively assessed by the AGE reader. This method uses the fluorescent properties of some AGE.

Part II elaborates on potential disturbing factors in the measurement of SAF. In chapter 3 the relation between SAF and short- and intermediate term glycemic variations is evaluated by both original data and a review of literature. We show that acute changes in glucose levels during an oral glucose tolerance test, even in patients with impaired glucose tolerance, do not influence SAF. AGE rich meals result in a transient postprandial rise in SAF of 10% 2-4 hours later, probably caused by the AGE content of the meal. In type 1 diabetes mellitus major intermediate term improvements of glycemic control as

depicted by multiple HbA1c measurements were associated with lower skin AGE levels. In a well-controlled, stable type 2 diabetes mellitus cohort, only a weak correlation was found between SAF and HbA1c. In both studies, skin AGE content/SAF levels predicted diabetic complications with an accuracy superior to that of HbA1c. SAF also had superior sensitivity to either fasting glucose or HbA1c in diagnosing IGT and diabetes mellitus.

Chapter 4 assesses the influence of potential disturbing factors in SAF measurement. Dark pigmentation of the skin is a well-documented limitation in the SAF measurement. Improvements in the autofluorescence reader however have led to a an extension of reliable SAF measurement in darker skin to reflectance levels of approximately 6% (up to Fitzpatrick skin type 5). We show that measurement of SAF is also strongly affected by several skin creams, causing a falsely increased SAF. This effect was often not fully corrected by alcohol swabs and washing with soap and may persist for many days. Marked hyperemia and vasoconstriction also influence SAF, respectively giving a decreased and increased measured SAF. Therefore it is strongly advisable to avoid these potential error sources.

Part III focuses on SAF, as an estimate of AGE accumulation, in atherosclerotic disease in different vascular beds. In chapter 5 we present the first report of SAF levels in patients with carotid artery disease. SAF was higher in the 56 patients with carotid artery stenosis compared to a healthy control group (matched for age and sex), especially in the younger age group. Coexistence of carotid artery stenosis and peripheral artery disease (PAD) resulted in an even higher SAF than in patients with carotid artery stenosis only. Regression analysis showed that age, smoking, diabetes mellitus, renal function and PAD were determinants of SAF, but carotid artery stenosis was not. The results suggest that increased SAF can be seen as an indicator of atherosclerotic burden.

In chapter 6 we investigate the hypothesis that SAF is elevated in peripheral artery disease (PAD) as suggested by the study presented in chapter 5. A case control study was performed in 492 patients with PAD and 164 controls, matched for age and presence of diabetes mellitus. SAF was higher in patients with peripheral artery disease compared to controls. PAD patients with cardiovascular co-morbidity had a higher SAF as compared to those without, also after correction for confounders. Regression analysis showed that age, smoking, diabetes mellitus, chronic kidney disease and a history of cerebrovascular disease or AAA were independently associated with an increased SAF in the patients with PAD. This study proves that SAF, as a measure of tissue AGE deposition, is considerably increased in patients with PAD as compared to controls. This increase is independent of the presence of cardiovascular risk factors or other established cardiovascular disease although these conditions are associated with a further increase of SAF. These findings

underscore the importance of AGE in PAD, irrespective of the presence of diabetes and renal insufficiency.

Chapter 7 presents the baseline cross-sectional data of a prospective study that aims to confirm the association between SAF and diabetic complications in patients with type 2 diabetes in a multicenter secondary care setting. We analysed 563 subjects with type 2 diabetes mellitus followed in 5 Dutch hospitals. SAF was elevated compared to historical age-matched healthy controls. SAF levels showed a graded increase with the presence and severity of diabetic micro- and macrovascular complications. Regression analysis showed that age, duration of diabetes, serum creatinine, gender and SAF were independent determinants of macrovascular complications. The level of SAF was determined by age, smoking, serum creatinine and macrovascular complications. This study confirms that SAF is increased in type 2 diabetic patients and has a graded relation with micro- and macrovascular complications. Also, SAF is an independent determinant of macrovascular complications, on top of classical risk factors.

In summary, this thesis has two major messages. First of all, it shows several factors that may disturb SAF measurement and thus should be avoided. Secondly, it presents evidence that SAF, as a measure of AGE accumulation, is consistently elevated in atherosclerosis in different vascular beds, irrespective of the presence of diabetes mellitus and renal insufficiency.

DISCUSSION AND FUTURE PERSPECTIVES

The role of AGE, their accumulation in tissue and its deleterious consequences have been partly elucidated as depicted in Part I. However, some of the effects are undoubtedly still unknown or incompletely understood. Partly, we understand the negative consequences of AGE, but the body might counteract the negative consequences of AGE by protective mechanisms. What these protective mechanisms are and how they work largely remains to be elucidated. Research data suggest that an increased circulating soluble receptor for AGE (sRAGE) acts as a decoy receptor.[1] Binding of AGE to sRAGE would facilitate clearance and also prevents activation of cellular receptors of AGE which would cause pro-inflammatory intracellular signals.[1] Genetic influences also may play an important role. In patients with rheumatic arthritis, polymorphisms of the receptor for AGE (RAGE) influence levels of sRAGE and are associated with disease activity and ischemic heart disease (submitted data by Hans Nienhuis). Some polymorphisms of RAGE seemed protective against severe rheumatic disease activity and ischemic heart disease. Similarly in systemic lupus an association was found between certain RAGE polymorphisms and disease severity in lupus nephritis.[2] However, no association of *RAGE* gene polymorphisms with type 2 diabetes mellitus, diabetic retinopathy and nephropathy was found by Kang et al.[3] Theoretically, protective mechanisms that inhibit AGE formation in the first place may also exist. Up-regulation or increased expression of antioxidant mechanisms may be triggered by increased serum AGE. The enzyme glyoxalase 1 (GLO1) is a known defence mechanism against the formation of AGE out of methylglyoxal/glyoxal. GLO1 breaks down methylglyoxal and glyoxal, thereby detoxifying these precursors of AGE. Polymorphisms in the GLO1 gene were, however, not associated with the prevalence of hypertension, markers of atherosclerosis, renal function and AGE. These polymorphisms are only weakly associated with pulse pressure and systolic blood pressure in two Dutch cohorts of patients with normal glucose metabolism, impaired glucose metabolism and type 2 diabetes mellitus.[4]

Cells with large accumulation of AGE may trigger a self-destructing mechanism like apoptosis, as suggested by some researchers.[5,6] Weather epigenetic changes are of influence and weather AGE maybe directly induce epigenetic changes has yet to be explored. Hopefully, future research will give more insight into such mechanisms.

What is the best method to determine AGE accumulation in tissue? Often, the specific AGE content in skin biopsy is regarded as the golden standard. However, this method has several flaws as a golden standard. First of all, determination of a single specific AGE is not representative for the whole spectrum of AGE. Measurement of several specific AGE in tissue gives a better estimate, but still represents only a selection of known AGE. Also, when measuring several AGE, how to weigh the different AGE?

Therefore, there are limitations to tissue AGE content as a golden standard for AGE accumulation in tissue. The need for skin biopsies and for elaborate and expensive laboratory assays also makes wide use in medical practice impossible. Measurement of skin autofluorescence with the AGE Reader is a much easier and non-invasive method to estimate AGE accumulation in tissue.[7] As explained in the introduction of this theses, multiple validation studies have shown convincingly and consistently that SAF has a strong correlation with both fluorescent and even non fluorescent AGE content in skin biopsies. Skin AGE content explained the major part of the variance (up to 76%) in the SAF signal in a pooled analysis of three validation studies.[8,7] Skin autofluorescence can therefore be used as an indirect, noninvasive estimate of AGE accumulation in tissue.[7] Still, there are pitfalls: the fluorescent properties detected by the AGE reader are not specific for AGE. NADPH has the same autofluorescent properties and, therefore, may confound the estimation of AGE by autofluorescence. Haemoglobin and melanin absorb the UVA light of the AGE reader, possibly interfering with measurement. Bilirubin also absorbs UVA light and, therefore, hyperbilirubinaemia may also interfere with SAF measurements. With all these limitations, SAF still is a predictor of mortality and complications in diabetes and renal failure.[9-12] In conclusion, there remain many difficulties and obstacles in reliably measuring tissue accumulation of AGE, but so far skin autofluorescence is the most practical and patient-friendly method available.

Part II shows that several precautions need to be taken to ensure a reliable SAF measurement. Use of skin creams on the volar forearm in the 2 weeks before SAF measurement should be avoided. Extreme dermal hyperaemia or vasoconstriction on the measuring site must be avoided. As a 10 % rise of SAF measurements can be seen 2-4 hours post oral exposure to a large AGE load we recommend a fasting measurement to improve accuracy. In previously performed studies these precautions were insufficiently taken into account. Even so, these studies showed a predictive value for mortality, microvascular and macrovascular complications.[10-13] Predictive results of the AGE reader can only improve when these precautions are conscientiously taken. The need for these (easily taken) measures does not negatively affect the possibilities of the AGE Reader for a broad application in the future. For blood pressure measurements, similarly, standardized conditions are taken into account to avoid large fluctuation of values.

The AGE reader is also still in further development, and continuing software improvements may also lead to increasing reliability of SAF measurement. For example, error detection for external light, and automatic warnings by the system for probable unreliable measurements due to use of skin creams, skin tanners and sunscreen blockers (as identified by algorithms using SAF and skin reflectance levels) will soon be implemented.

Part III presents proof that SAF, as an indirect estimate of AGE accumulation, is elevated in atherosclerotic disease in different vascular beds. Chapter 7 shows that SAF is an independent determinant of macrovascular complications in patients with type 2 diabetes, on top of classical risk factors. Chapter 5 describes that SAF levels are increased in patients with carotid artery disease and peripheral artery disease, irrespective of diabetes or renal failure. In chapter 6 we show that SAF is elevated in patients with peripheral artery disease. Also, SAF increased even further with cardiovascular co-morbidity (AAA or CVA), indicating that SAF may be seen as a cumulative score of atherosclerotic burden. These findings underscore the importance of AGE in atherosclerotic disease, irrespective of the presence of diabetes and renal insufficiency. Moreover, as SAF increases further with Also, earlier studies showed that a high SAF predicted cardiovascular mortality in patients with type 2 diabetes and in patients on haemodialysis.[10,13] SAF also proved to be elevated in chronic inflammatory diseases like rheumatoid arthritis, systemic lupus erythematosis and granulomatosis with polyangiitis.[14-16] Interestingly, these diseases are also characterized by a high risk of premature atherosclerotic events. A relation was found between small artery elasticity and SAF in patients with SLE, again suggesting the role of AGE accumulation in atherosclerosis.[17] A logical conclusion: SAF appears to be a marker of vascular damage. AGE accumulation is however non-specific for any disease: glycemic stress and oxidative/carbonyl stress can result from a wide range of diseases and medical situations.

Some acute diseases can also temporary cause elevated levels of SAF, like sepsis and acute myocardial infarction.[18,19] This temporarily elevation of SAF is probably caused by high oxidative stress and high serum AGE, but possibly not by accumulation of AGE in tissue. This contrasts with chronic diseases where an increased SAF indicates high tissue AGE and appears to reflect atherosclerotic burden.

Maybe the spectrum of diseases that exhibit an elevated SAF with broaden in future years. Theoretically one could think of chronic diseases like Morbus Crohn, colitis ulcerosa, COPD, psoriasis or severe eczema, because of the chronic generalized inflammation. Another group of patients that have endured a great amount of oxidative stress are survivors of cancer who had long-term chemotherapy. Young patients who survive Hodgkin's disease are known to have a high risk of future coronary events. It is thought that this is the result of chemical damage of chemotherapy and radiation to the arterial vessel wall. However, maybe AGE accumulation plays an important role. Furthermore, AGE accumulation in DNA or transcription factors could theoretically be a direct cause of cancer. It would be interesting to study SAF levels in these groups of patients. Even so, it may well be that out of all long lived tissues that can be damaged by AGE, the vascular wall endures most damage and causes most morbidity and mortality due to continuous exposure to (external) factors transported in the blood stream. In that

case, all chronic diseases that result in an increased AGE accumulation would probably also exhibit premature vascular events.

Normally, cardiovascular risk is estimated by the classical risk factors: age, blood pressure, lipid profile, smoking behaviour and diabetes mellitus. However, evidence is growing that SAF adds information on top of the classical risk factors in predicting cardiovascular events. In patients with diabetes mellitus type 2 SAF added information in predicting cardiovascular mortality on top of the UKPDS risk score.[11] Cardiovascular risk in inflammatory diseases like rheumatoid arthritis, SLE and GPA cannot be explained by the classical risk factors, while increased SAF is associated with increased vascular damage in these groups of patients.[20] Prospective follow-up studies need to further determine the value of SAF as predictor of cardiovascular events in different groups of patients (other than diabetes mellitus or renal failure). Such a study is now being executed in a large cohort of patients with peripheral artery disease.

Another question that may be answered by future research is whether consecutive SAF measurements can be used in follow-up. For example, can a repeated SAF measurement after 5-10 years be used to monitor effect of therapy or progression of atherosclerotic disease? Theoretically, a repeated measurement within 5 years may not be useful, because AGE accumulation in tissue is a slow process over many years. However, quite recently, Arsov et al showed in an extreme model of increased AGE accumulation in patients on haemodialysis, that the 1-years change in SAF was a stronger predictor of mortality than the baseline SAF (Arsov, Artific Organs, in press).

The next issue to be solved is what to do after determining that there is increased AGE accumulation. Maybe patients with a high SAF may benefit from more aggressive treatment of classical risk factors (i.e. lower LDL-cholesterol and blood pressure targets), similar to patients with diabetes mellitus or renal insufficiency. It is now well accepted that cardiovascular risk reduction may be achieved by interventions not necessarily focused on the abnormal risk predictor itself. For example in diabetic or hypertensive persons lipid lowering is effective in cardiovascular risk reduction, even when lipid levels are not elevated. This should be subject to an intervention study. Furthermore, inhibition of AGE formation, breakdown of AGE-crosslinks, blocking the receptor for AGE may become treatment targets in cardiovascular disease. The AGE formation inhibitor benfotiamine and the presumed crosslink breaker alagebrium have been tested, but provided mixed results. In diabetic nephropathy, benfotiamine reversed increased urinary albumin excretion in patients with type 2 diabetes and microalbuminuria in some studies, but failed to do so in another study.[21,22] Alagebrium had a positive effect of isolated systolic hypertension in one study,[23] while no effect was seen in on systolic of diastolic function or exercise tolerance in heart failure.[24] The latter study was of relatively short duration (36 weeks), which may explain the negative results. Soluble

RAGE analogues are also under development to scavenge circulating AGE. So far none of these drugs have yet been introduced for clinical use because of mixed results on efficacy and safety concerns. Interestingly, drugs that are currently prescribed to treat diabetes mellitus, hypertension and hypercholesterolemia may have effects on AGE. For example, Angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB) have been shown to reduce the formation of AGE and affect the receptor for AGE.[25-28] Also, ACE inhibitors appear to upregulate the soluble receptor for AGE.[26] Statins also have also been shown to reduce the receptor for AGE as well as serum levels of AGE which may explain their known pleiotrophic effects.[29-33] Reducing the intake of AGE in food and smoke also is a possible target to lower AGE accumulation. A diet low in AGE is unpalatable, however.

Concluding, there is a lot of work yet to be done in the field of AGE accumulation and SAF. It makes me very humble about the data I present in this thesis. Socrates expressed my feelings when he said: the more I learn, the more I learn how little I know.

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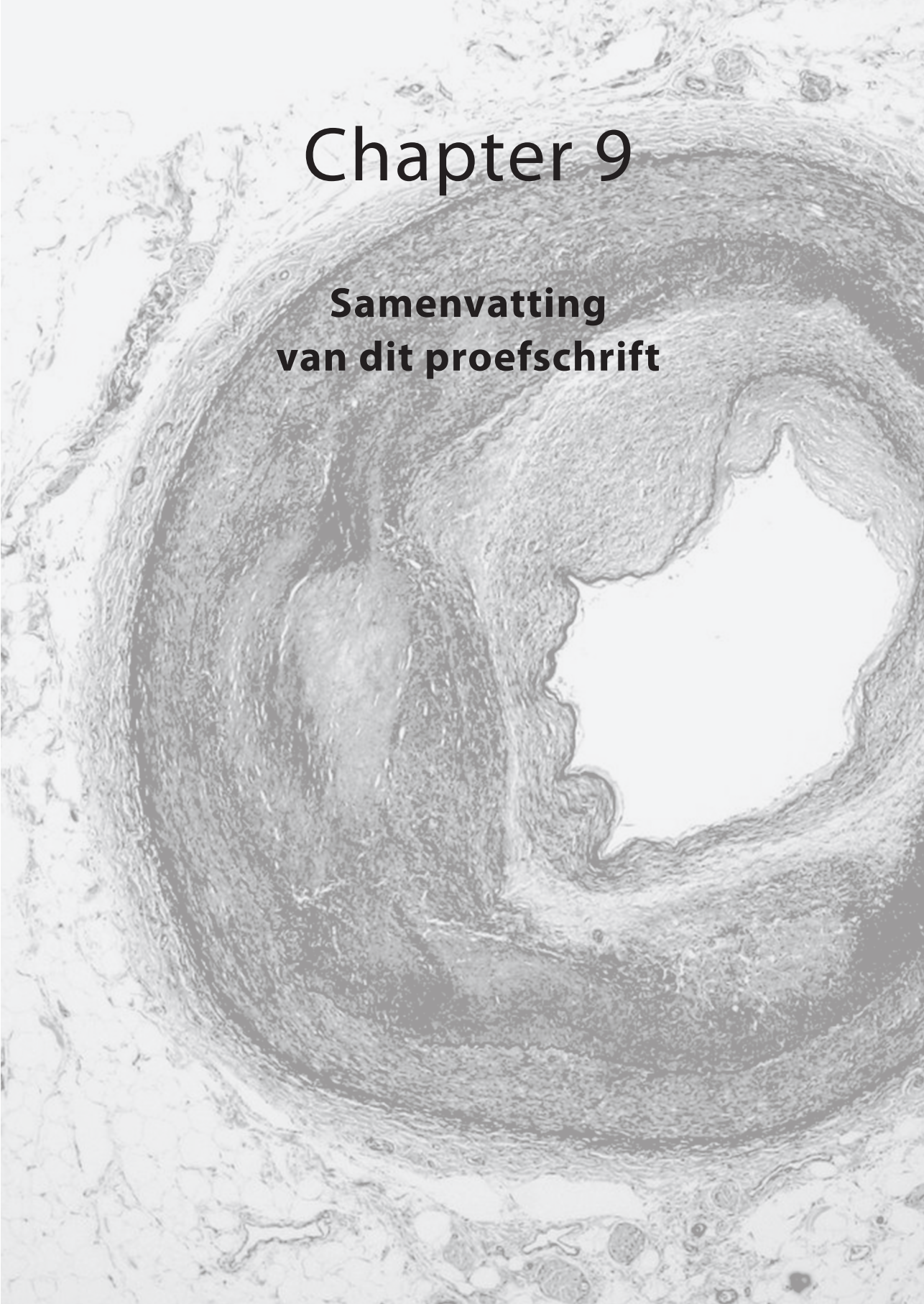


Chapter 9

Samenvatting van dit proefschrift

Deel 1 geeft een overzicht over de rol van advanced glycation end products (AGE), waarbij in hoofdstuk 2 de nadruk ligt op hun rol bij nierfalen. AGE ontstaan als gevolg van versuikering en/of oxidatie van eiwitten, vetten en DNA. AGE ontstaan in feite bij alle mensen als onderdeel van het normale verouderingsproces. Ze geven schade aan weefsels doordat ze onomkeerbare verbindingen aangaan met allerlei eiwitten die daardoor misvormd raken en niet goed kunnen functioneren. Ze kunnen ook verbindingen vormen tussen eiwitten, bijvoorbeeld in het bindweefsel van de vaatwand, die daardoor zijn elasticiteit verliest. Verder activeren AGE een specifieke celmembraan receptor (RAGE genoemd) die binnen de cel tot allerlei negatieve processen leidt. Als AGE ontstaan in weefsel met een korte levensduur, wordt de AGE vanzelf afgebroken, zodat de schade die de AGE kan aanrichten beperkt is. Dit is anders bij AGE die ontstaan in eiwitten of weefsels die 15 tot 20 jaar of zelfs levenslang meegaan. Bindweefsel in de huid en vaatwand hebben bijvoorbeeld een levensduur van 10-20 jaar. Opstapeling van AGE in weefsels met een lange levensduur treedt in verhoogde en versnelde mate op bij patiënten met diabetes, chronisch nierfalen en chronische ontstekingsziekten (bv reumatoïde artritis). Bij diabeten leidt de hoge suikerspiegel in het bloed tot verhoogde versuikering van eiwitten en dus een verhoogde AGE stapeling. Bij nierfalen leidt het slechter uitscheiden van voorlopers van AGE tot een stapeling van deze schadelijke stoffen. Bij ontstekingsziekten als reumatoïde artritis veroorzaken schadelijke stoffen van de ontstekingscellen voor versnelde AGE vorming. Dit verklaart waarom deze patiënten groepen een hoog risico op allerlei chronische complicaties hebben en vroegtijdig overlijden. Eigenlijk kan AGE opstapeling in weefsels met een lange levensduur gezien worden als eindorgaanschade, een metabool geheugen van schade door oxidatie en versuikering.

Hoe kan deze kennis gebruikt worden in de praktijk? Hoe kun je AGE stapeling meten? Het betreft namelijk een verzameling van schadelijke eindproducten, niet één specifieke stof. Specifieke AGE met een bekende structuur kunnen met speciale methoden gemeten worden in bloed. Dit geeft echter een momentopname van de hoeveelheid AGE in bloed, terwijl we geïnteresseerd zijn in de hoeveelheid stapeling van AGE in weefsel. Een mogelijkheid om weefsel stapeling te meten is door een huidbiopt te nemen en daarin de hoeveelheid van een AGE bepalen. Het nemen van een huidbiopt maakt routine gebruik in de praktijk echter onmogelijk. Sinds 2006 is er een niet belastende en eenvoudige methode beschikbaar om een schatting te maken van AGE stapeling in de huid: de Autofluorescentie Reader. Dit apparaat maakt gebruik van het fenomeen “autofluorescentie”. Dit houdt in dat bij een belichting met UVA stralen deze AGE een andere, herkenbare lichtfrequentie uitstralen. Met de Autofluorescentie Reader wordt een meting gedaan op de onderarm door belichting met UVA stralen.

Een spectrometer analyseert vervolgens de herkenbare lichtfrequentie die de AGE terugsturen (golflengte 420-600 nm) en het weerkaatste UVA licht. Aan de verhouding hiertussen wordt de autofluorescentie berekend. Uit meerdere validatiestudies blijkt dat deze autofluorescentie (SAF) een goede schatting geeft van de hoeveelheid AGE stapeling in de huid. SAF blijkt bij diabetes patiënten verhoogd te zijn en ook een voorspellende waarde te hebben voor vroegtijdig overlijden en het optreden van microvasculaire complicaties (langetermijns schade aan kleine bloedvaatjes zoals oogschade, nierschade en zenuwschade) en macrovasculaire complicaties (aderverkalking van grote bloedvaten waardoor hartinfarct, beroerte of vernauwde slagaders naar de benen). Bij hemodialyse patiënten is de SAF erg verhoogd en voorspelt het overlijdensrisico. Na een niertransplantatie daalt de SAF enigszins maar blijft toch fors verhoogd ten opzichte van normaal. Een hoge SAF is een sterke risicofactor voor het falen van het niertransplantaat.

Deel II onderzoekt meerdere factoren die de SAF meting kunnen verstoren. In hoofdstuk 3 wordt de relatie besproken tussen SAF en korte termijns en middellange termijns variaties in glucose waarden. Korte termijns variaties in glucose, uitgelokt door een gestandaardiseerde suiker belasting test (OGTT) bleken geen invloed te hebben op de gemeten SAF, zelfs bij neiging tot diabetes. Maaltijden die veel AGE bevatten gaven een tijdelijke stijging van de SAF van 10% 2 tot 4 uur na de maaltijd. In een studie bij type 1 diabeten resulteerden drastische verbetering van glucose regulatie op middellange termijn (meerdere opeenvolgende HbA1c waarden) in een lagere AGE hoeveelheid in huidbiopten. In een cohort van goed ingestelde type 2 diabeten werd slechts een zeer zwakke relatie gevonden tussen SAF en HbA1c. In beide studies voorspelde de hoeveelheid AGE in de huid (gemeten via huid biopten dan wel SAF) het risico op toekomstige micro- en macrovasculaire complicaties beter dan het HbA1c. Verder heeft SAF een hogere sensitiviteit dan nuchter glucose of HbA1c om beginnende diabetes te diagnosticeren.

Hoofdstuk 4 bespreekt welke factoren van de huid de SAF meting kunnen verstoren. Een donker gepigmenteerde huid maakt een SAF meting moeizaam. Door recente verbeteringen in de autofluorescentie reader wordt de meting ook bij een donkere huidpigmentatie steeds betrouwbaarder. Wij toonden aan dat vele huid crèmes (met name dagcrème, zonnebrandcrème en zelf-bruinende crème) en extremen in doorbloeding van de huid de SAF waarde sterk beïnvloeden. Deze verstorende factoren moeten vermeden worden om een betrouwbare SAF meting te waarborgen. Vochtigheid van de huid beïnvloedde de SAF meting niet.

In deel III staat centraal dat AGE stapeling een grote rol lijkt te hebben in verschillende uitingen van aderverkalking (atherosclerose)

Hoofdstuk 5 laat zien dat SAF, als schatting van de AGE ophoping in de huid, verhoogd is bij patiënten met carotis stenose (vernauwing van een halsslagader) en perifeer vaatlijden (vernauwing van vaten naar de benen). SAF was fors hoger bij de 56 patiënten met carotis stenose vergeleken met de gezonde controle groep, vooral in de jongere leeftijdsgroep. Patiënten met zowel carotis stenose én perifeer vaatlijden hadden weer een hogere SAF dan patiënten met alleen carotis stenose. Bij regressie analyse bleek de SAF met name bepaald door leeftijd, roken, diabetes, nierfalen en perifeer vaatlijden. Deze resultaten suggereren dat SAF gezien kan worden als een maat voor uitgebreide atherosclerose.

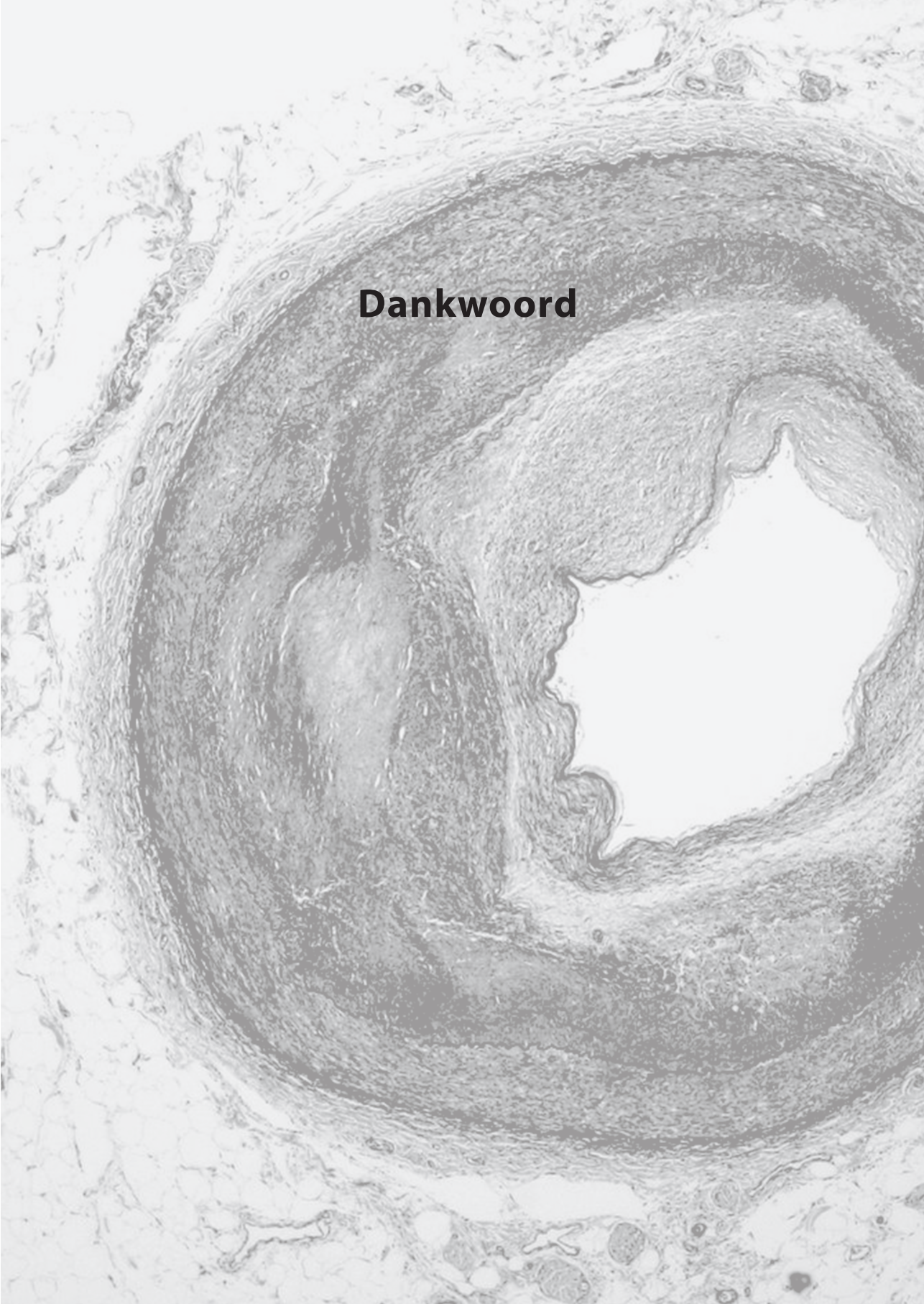
In hoofdstuk 6 onderzoeken we de hypothese dat SAF ook verhoogd is bij perifeer vaatlijden (PAD). In een case control studie van 492 patiënten met PAD en 164 controle mensen, gematched op leeftijd en diabetes, bleek SAF inderdaad verhoogd ten opzichte van de controle groep. Bij de patiënten met PAD werd een verdere stijging van de SAF gezien indien er ook sprake was van een doorgemaakte beroerte (CVA) of een verwijde buikslagader (aneurysma). Regressie analyse toonde dat leeftijd, roken, diabetes, chronisch nierfalen en een doorgemaakt CVA of AAA onafhankelijke samenhingen met een verhoogde SAF bij depatiënten met PAD. Deze studie bewijst dat SAF fors verhoogd is bij patiënten met perifeer vaatlijden ten opzichte van controle patiënten zonder vaat-lijden, onafhankelijk van de traditionele risicofactoren voor hart- en vaatziekten. Hoe meer aanvullend vaatlijden naast het perifeer vaatlijden, hoe verder de SAF stijgt. Deze bevindingen bevestigen dat AGE een grote rol lijken te spelen in atherosclerose, losstaand van diabetes of nierfalen.

Hoofdstuk 7 presenteert de aanvangsdata van een in opzet prospectieve studie. We analyseerden 563 patiënten met type 2 diabetes die behandeld werden in 5 verschillende ziekenhuizen. SAF was fors verhoogd bij de diabetes patiënten ten opzichte van gezonde controle mensen. Met toename en ernst van de micro- en macrovasculaire complicaties verhoogde de SAF ook stapsgewijs. Bij regressie analyse bleek SAF een onafhankelijke determinant voor macrovasculaire complicaties. De studie bevestigt dat SAF verhoogd is bij patiënten met diabetes type 2 en dat er een sterke relatie bestaat tussen SAF en diabetische complicaties.

Kortom, dit proefschrift heeft 2 belangrijke conclusies. Allereerst zijn er meerdere factoren die de SAF meting beïnvloeden. Deze storende factoren moeten vermeden worden om een betrouwbare meting te waarborgen. Ten tweede spelen AGE een grote rol in het ziekteproces van aderverkalking. SAF, als maat voor AGE stapeling, is consequent verhoogd bij meerdere uitingen van atherosclerose.

DISCLAIMER

The articles presented in this thesis may have minor textual differences with the published version in the medical journals. Besides these incidental differences in terminology or sentence structure, there are no differences in content.



Dankwoord

Het afronden van dit proefschrift is een gedenkwaardig moment voor mij. Hierbij kijk ik met dankbaarheid terug op de hulp die ik van vele mensen ontving in het bereiken van dit doel. Een aantal mensen verdienen specifiek een woord van dank.

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Allereerst grote dank aan Dr. J.D Lefrandt. Tijdens het hele traject heb ik van hem veel steun, positieve stimulans en inzet ontvangen. Beste Joop, je bent altijd blijven meedenken over het beloop van mijn promotietraject en kwam hierbij meerdere malen met geniale ideeën aanzetten. Ook dank voor je hulp bij het maken van figuren in grafische programma's waar ik (als Nederlandse) toch echt geen kaas van gegeten had. Joop, jij was echt onmisbaar. Door jou bleef ik het enthousiasme en de motivatie houden om door te gaan met het promotieonderzoek, ondanks dat dit naast de baan als internist in Heerenveen lastig was.

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Beste Rijk, jouw steun en vertrouwen in mij waren erg belangrijk voor mij. Dit des te meer omdat ik grote bewondering heb voor je grote gedrevenheid als medicus en wetenschapper. Eén opmerking zal ik nooit vergeten. Je zei: "als jij jouw tanden ergens in zet, dan laat je niet meer los tot het klaar is, dus het komt goed met dat promotieonderzoek. Je bent wat dat betreft gewoon een tijger." Dat compliment heeft mij ook geholpen om dit ook waar te maken. Toen een collega internist uit Heerenveen plotseling ziek werd en uitviel, werd de werkdruk in Heerenveen abrupt groter en moest ik meer werken om in de crisissituatie te helpen. Rijk, fijn dat jij die situatie begreep en toestond dat ik ten koste van mijn promotieonderzoek meer ging werken in Heerenveen. Een gentlemen's agreement waar mijn collega's in Heerenveen je dankbaar voor waren. Prof.dr. Zeebregts, beste Clark, mijn dank voor jouw begeleiding en hulp bij het carotis artikel. Ik heb bewondering voor je snelle reacties en daadkrachtige optreden in het maken én submitten van de artikelen. Dit zelfs tijdens vakanties en weekenden, waarbij ik me zelfs een reactie herinner vanaf het vliegveld.

Tijdens de onderzoeken was samenwerking met meerdere personen essentieel. In het AURORA onderzoek is nauw samengewerkt met Peter Oomen uit het MCL Leeuwarden, Manuel CastroCabezas uit het Sint Franciscus Gasthuis te Rotterdam, Jan Jager uit het Diakonessenhuis Meppel en Teri Brouwer uit het Onze Lieve Vrouwe Gasthuis te Amsterdam. In het OLVG heb ik goede hulp gekregen van Lucy Schrijnders en Rosa Regez en in het SFG van mevrouw Ferwer. Het artikel "External factors influencing skin autofluorescence" heeft tot stand kunnen komen door samenwerking met Reindert Graaff en Marten Koetsier. Verder is hulp van de firma DiagnOptics van grote waarde geweest. Dank voor jullie medewerking!

In de werkkamer in het UMCG, initieel bij de arts-assistenten interne geneeskunde en later aan de andere kant van het schot bij de onderzoekers van de longziekten/ reumatologie heb ik mooie tijden beleefd. Marjan, Sake, Udo: ik heb jullie zeer gewaardeerd als collegae-fellows vasculaire geneeskunde. Samen hebben we een bijzondere tijd doorgebracht. Helen, bedankt voor je medewerking aan het carotis-artikel. Udo, aan jou specifiek nog dank voor jouw hulp bij mijn laatste artikelen. Je hebt het hart van een rasechte wetenschapper en academicus! Ruth, Susanne, Maartje, Eef, Gerald, het was gezellig op de onderzoekskamer en soms zelfs té gezellig! Dank voor een luisterend oor, begrip bij onderzoeksfrustraties en hulp bij allerlei statistiek probleempjes.

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Mijn fijne collega internisten uit ziekenhuis "Tjongerschans" Heerenveen wil ik laten weten dat ik de werksfeer, samenwerking en collegialiteit erg waardeer. Ook zij hebben altijd zeer positief gestaan ten opzichte van mijn promotietraject en gaven mij hiervoor de ruimte. Dit maakte het afronden van mijn promotieonderzoek terwijl ik al te Heerenveen als internist-vasculair geneeskundige werkte mogelijk.

Mijn paranimfen Annemieke Gras-Siero en Alice Mol ben ik dankbaar dat ze mij in het uur der waarheid terzijde wilden staan. Jullie zijn van grote waarde voor mij! Wat fijn dat we zulke bijzondere momenten met elkaar mogen delen.

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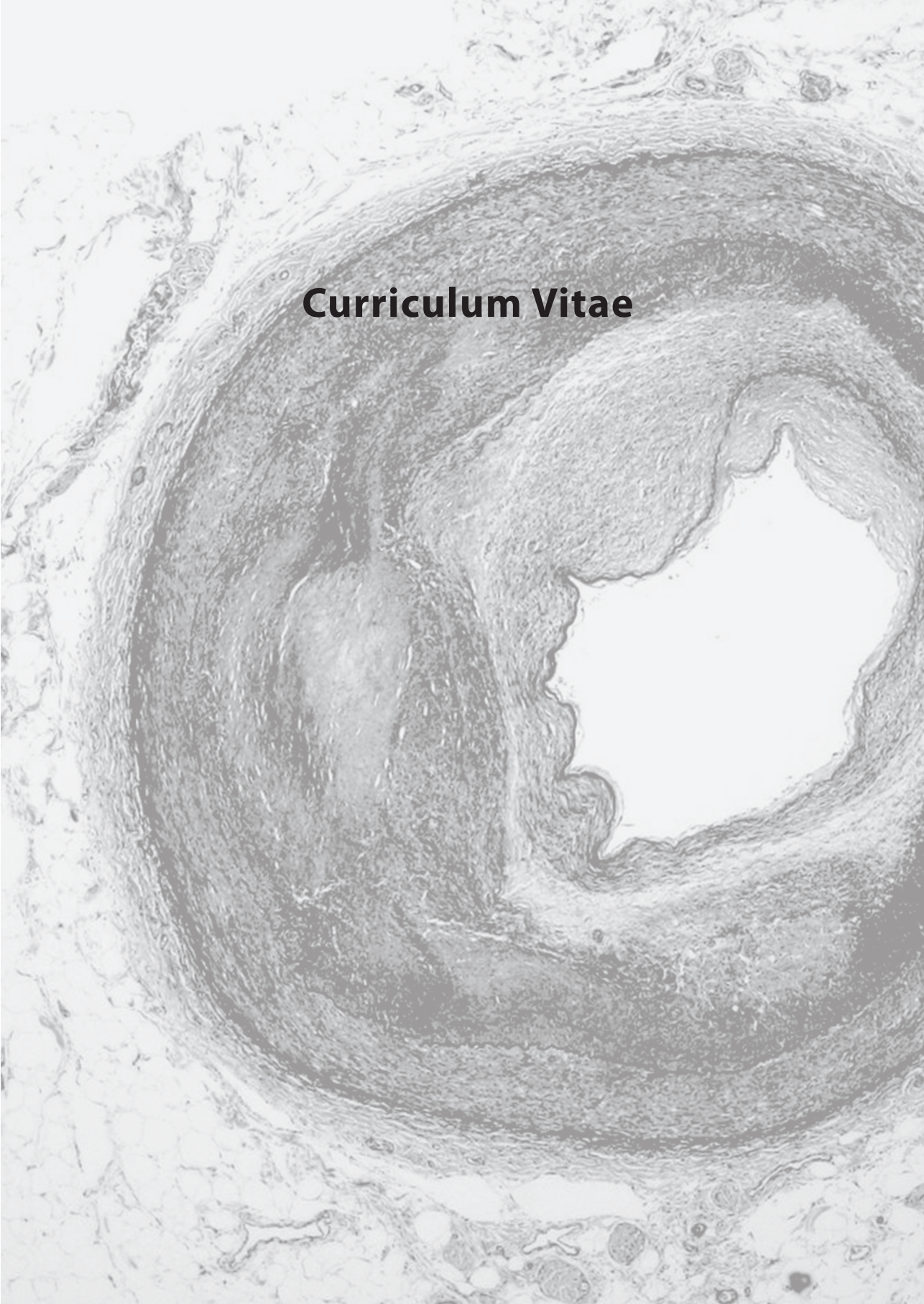
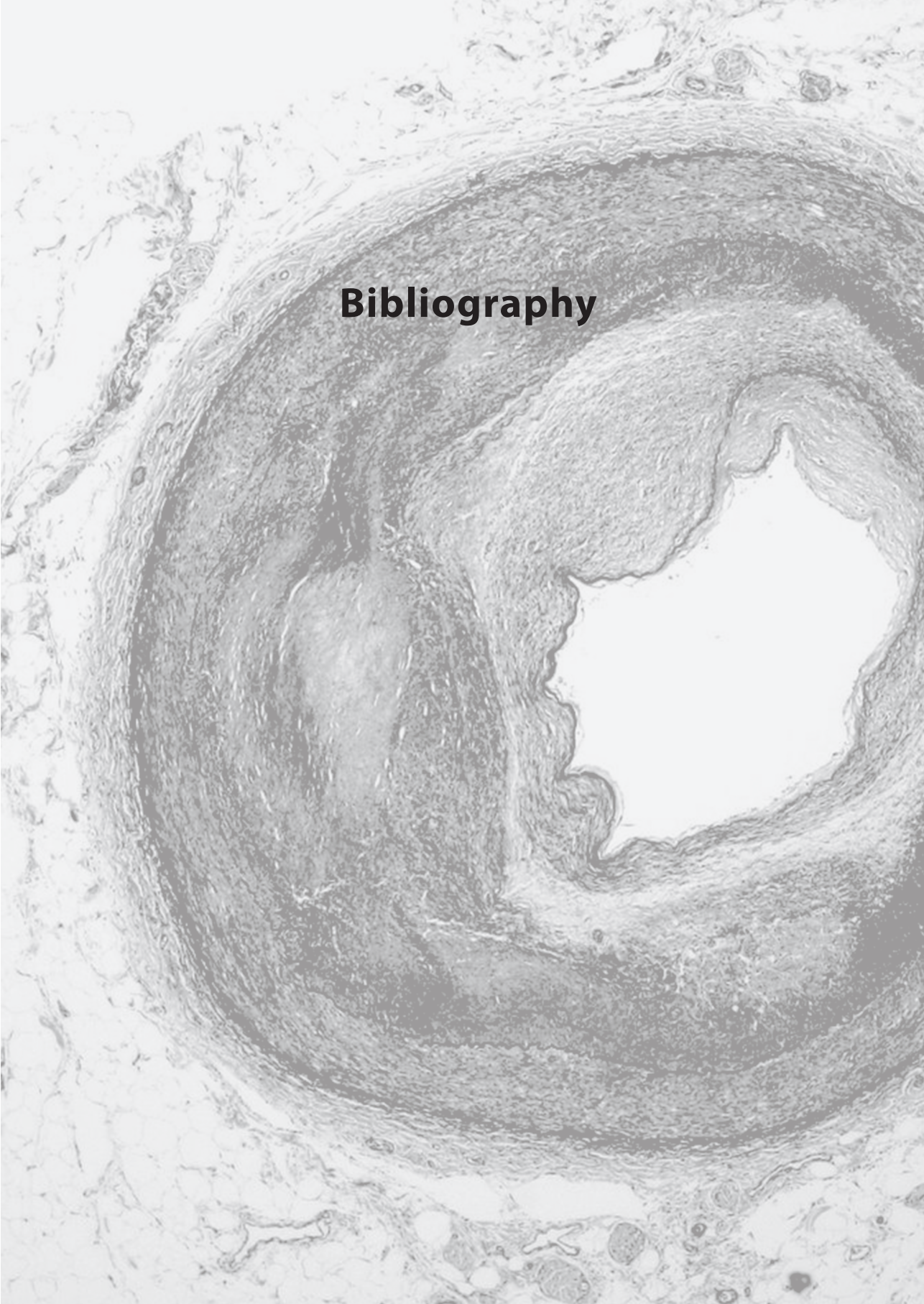


Curriculum Vitae

De auteur van dit proefschrift werd geboren op 25 januari 1977 in Ermelo. Zij volgde het atheneum op het Christelijk College "Groevenbeek" te Ermelo en behaalde in 1995 haar VWO diploma. Direct aanvolgend startte zij met de studie Geneeskunde aan de Universiteit Utrecht. Initieel volgde zij daarnaast ook enkele vakken Natuurkunde, onder andere fysica van de mens en relativiteitstheorie. Tijdens haar studie Geneeskunde ontstond interesse voor bijzondere casuïstiek en onderzoek wat resulteerde in enkele bescheiden publicaties. Alvorens in 2000 door te gaan met haar coschappen besteedde Marjon een jaar als bestuurslid van het Utrechtsch Studenten Concert waar zij al jaren met passie musiceerde als hoboïste. In 2002 behaalde zij haar doctoraal examen als arts. Vervolgens startte zij in 2003 haar opleiding tot internist met Prof. Dr. R.O.B. Gans als opleider. Ze deed in het MCL Leeuwarden en het Deventer Ziekenhuis ervaring op in de algemene interne geneeskunde. Wederom publiceerde ze enkele case-reports naar aanleiding van opvallende ziektegeschiedenissen. In november 2007 startte zij met de specialisatie Vasculaire Geneeskunde in het Universitair Medisch Centrum Groningen onder leiding van Dr. A.J. Smit (inmiddels benoemd tot bijzonder hoogleraar Vasculaire Geneeskunde). Tijdens deze specialisatie begon zij aan het promotietraject. November 2009 ontving ze de artsenbul. Per januari 2010 startte zij als jonge, enthousiaste internist in ziekenhuis "Tjongerschans" te Heerenveen waar zij een Vaatrisicopoli opzette. Per april 2011 is zij toegetreden tot de maatschap Interne Geneeskunde aldaar. Deelname aan het stafbestuur van ziekenhuis "Tjongerschans" volgde in april 2012. Die maand was vooral om een andere reden ook erg bijzonder: ze trouwde op 21 april met Richard Dam. Naast deze life-events bleef zij gestaag doorwerken aan haar promotieonderzoek die zij uiteindelijk in 2013 kon afronden.



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